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Effects, Particularly Anemia, Produced in Chicks by Growth in Their Yolk Sacs of Mouse Mammary Tumors

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INTRODUCTION

Heilman and Bittner (7) have reported that the serial transfer of mouse mammary tumors in the yolk sacs of fertile eggs is associated with their manifesting an increased lethal effect on the developing chicks. The first purpose of this study was to perform sufficient experiments of a similar nature to provide confirmation or denial of this finding. The second purpose was, if confirmation were obtained, to learn, from the study of the tissues and fluids of the chicks concerned, the cause or causes of their deaths. It was thought that precise information on this point might allow some proper inference to be drawn about the nature of the increased lethal effect, and that this in turn might be of some relevance to the general problem of malignancy.

MATERIAL AND METHODS

Fertile eggs from a pure strain of White Leghorns were incubated at 99.5° F. (wet bulb—83° F.). Yolk sacs were inoculated with mouse tumor tissue on the fourth day of incubation, essentially by the method of Taylor, Hungate and Taylor (14). Tumors resulting from this procedure were serially transferred in eggs. i.e., when the first tumors were obtained in eggs they were recovered and injected into other eggs, then when tumors grew in these they were injected into still other eggs, and so on. The details regarding the tumors so treated are given, with the observations made on them.

For primary inoculations of eggs, tumor tissue obtained directly from a mouse was minced, and then diluted to 5 times its volume with physiological saline. For subsequent egg inoculations, yolk sac tumor tissue was diluted to the same extent, not with saline, but with a mixture of yolk, amniotic fluid, allantoic fluid and blood from the egg in which the tumor was grown. This dilution was consistently adhered to except for a separate experiment which was performed to test the effects of different dilutions. The details of the dilutions employed in this separate experiment are presented later.

To obtain undiluted blood for hemoglobin and blood sugar estimations the shell over the air space was removed. The shell membrane so exposed was wetted to make it transparent. The large allantoic vein beneath it, then being visible, was grasped with forceps pushed through the membrane, and drawn out. The blood was taken from it with a 0.25 cc. syringe provided with a No. 25 gauge needle. Hemoglobin estimations were made with the Evelyn colorimeter by Evelyn's method. Because the chick's erythrocytes are nucleated, and hence difficult to count apart from leukocytes and thrombocytes, and because leukocytes and thrombocytes comprise a very insignificant proportion of the total blood cells, particularly at this stage of development, total blood counts were made and employed for color index determinations. Blood films were prepared and stained with Hasting's stain. Some blood sugar determinations were also made by the method of Miller and Van Slyke (11).

Sections of formalin-fixed tissues and organs of chicks were cut in paraffin and stained with hematoxylin and eosin.

OBSERVATIONS

1. ON THE ENHANCED LETHAL EFFECT OF TUMORS AFTER SERIAL EGG PASSAGE

Four tumors that we named and treated as follows were studied: ET, a spontaneous mam-

mary carcinoma from a C3H female mouse, was transplanted once into another C3H mouse and then into eggs in which it has been transferred 40 times to date; ECT, a spontaneous mammary tumor from a female C3H mouse, was injected directly into eggs, where it has undergone 8 transfers; EAT 1, a spontaneous mammary tumor from a mouse of the A strain, was injected directly into eggs, where it underwent 12 transfers before it was lost; EAT 2, a spontaneous mammary tumor from a female mouse of the A strain, was transplanted once into another A strain mouse and then into eggs, where it has undergone 41 transfers.

The *early* (first 10 days of incubation) mortality rate of chicks varied neither with the number of times the tumor had been transferred in eggs nor with the particular tumor grown; it remained remarkably constant (between 21 and 25 per cent) except for a period during which the war-time motor in our incubator became inefficient with regard to circulating the air and so caused a general increase in the mortality rate of both inoculated and normal eggs, a state of affairs that returned to normal after the installation of a new motor.

Cultures in thioglycollate medium (Difco Bacto-Fluid) showed that only about 3 per cent of the early deaths could be attributed to bacterial infection. Operative interference, it is presumed, was responsible for the remainder. Our results in this respect seem to be similar to those of King, Ball and Menefee (9) who observed some early deaths not due to contamination.
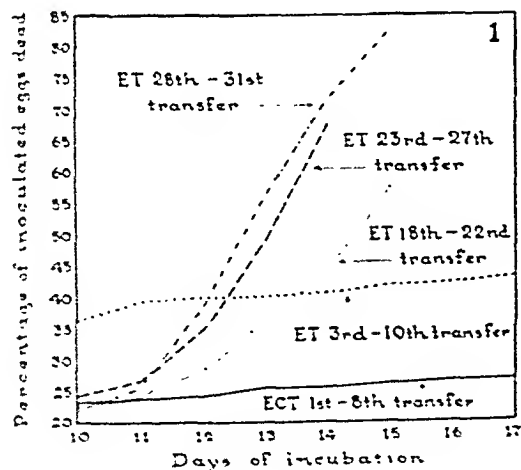
The *late* (from the 11th to 17th day of incubation) mortality rate was, in the early egg transfers of all 4 tumors, consistently low. But, with the continued serial transfer of tumors in eggs, the late mortality rate became progressively greater and the time of death progressively earlier.

Our observations regarding these two points on ET, the tumor that we have studied most, are recorded graphically in Fig. 1. It is to be noted that the results from several consecutive transfers, rather than from each transfer, have been plotted; this is to facilitate the reading of the graph and also to assure that each curve was plotted from a large number of eggs. It is to be noted also that no results are plotted for the 11th to 17th transfers; this omission is due to the incubator trouble described above, which, being difficult to diagnose, was not remedied until the 16th transfer, and resulted in too few eggs being carried through this period to provide figures of statistical significance. However, in the instance of normal eggs, it was observed that the incubator defect affected the early rather than the late mortality rate, hence, as eggs inoculated after the 11th transfer began to die in increasing numbers in the later stages of incubation, and as they revealed pathological stigmas found afterward to be characteristic of the increased lethal effect of the tumor, it seems justifiable to assume that the first signs of the enhanced lethal effect of ET occurred after its 11th transfer and, as is indicated in Fig. 1, was well established by the 18th transfer.

Fig. 1. — Effect of number of times tumors have been transferred in eggs on their lethal effect. ECT 1-8 was plotted from 164 eggs, ET 3-10 from 219 eggs, ET 18-22 from 599 eggs, ET 23-27 from 667 eggs, and ET 28-31 from 564 eggs.
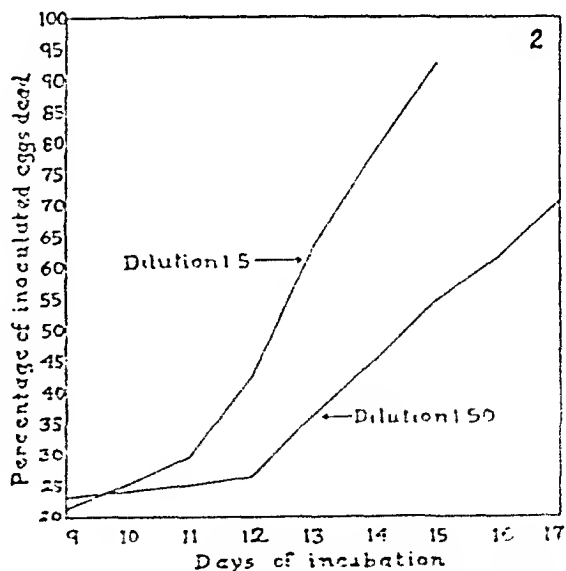
Fig. 2. — Effect of amount of tumor tissue (derived from tumors of many serial passages) with which eggs are inoculated on lethality of tumor subsequently developing.

In the serial transfer of ET in eggs, 99 per cent of the inoculated eggs developed yolk sac tumors.

The late mortality rate of eggs inoculated with ECT, another C3H tumor, is also shown in Fig. 1. From our experience its curve may be taken as typical of the late mortality rate of eggs inoculated with mouse mammary tumors that are in their early egg transfers.

The late mortality rate of eggs inoculated with EAT 1, and an A strain mammary tumor, remained very low for 8 transfers, but thereafter rose rapidly until the 12th transfer, at which point the tumor was lost because the eggs were not harvested soon enough. A contributing factor was the incubator trouble which, by causing an increased number of deaths in the early days of incubation, limited the number of eggs from which transfers could be made.

The late mortality rate of eggs inoculated with EAT 2, our second A strain tumor, remained very low for 20 egg transfers. From this point, however, it increased and now, in its 41st transfer, is such that eggs must be harvested on the 13th or 14th day of incubation in order to continue its further passage.

Cultures showed that only 1.4 per cent of the late mortalities were due to bacterial infection.

2. On the Relation of the Amount of Tumor Tissue Injected to the Lethal Effect of the Tumor

In a special experiment made to investigate this point, the lethal effect of tumors grown from 0.1 cc. of tumor tissue was compared with that of those grown from 0.01 cc.

Between the 35th and 40th transfers of ET, each tumor harvested was diluted to 5 times its volume with a mixture of yolk, amniotic fluid, allantoic fluid, and blood obtained from the chick in which the tumor was grown. Part of this 1-in-5 dilution of tumor tissue was then diluted further to 10 times its volume with the same fluids. By this method a 1-in-5 and a 1-in-50 dilution of each tumor harvested was prepared for inoculation into eggs. Two hundred eggs were each injected with 0.5 cc. of the 1-in-5 dilution and 224 with 0.5 cc. of the 1-in-50 dilution.

All the eggs that died or were harvested between the 10th and 17th days of incubation were examined for tumors. Ninety-nine per cent of those inoculated with the 1-in-5 dilution, and 93 per cent of those with the 1-in-50 dilution had tumors.

After subtracting the number of eggs that did not develop tumors, from the total number inoculated with each dilution, a graph (Fig. 2) was constructed to compare the percentages of late deaths of eggs inoculated with the two dilutions. Allowance was made for the few live eggs that were harvested on the 13th and 14th days to obtain tumor tissue for further transfer of the tumor. Since tissue from each tumor used in this experiment was injected into eggs in two different dilutions, it may be assumed for a basis of comparison between the results obtained from the two dilutions, that the quality of the tumor tissue used was a constant. Further, since the amount of the mixture of fluids from the chick was the same in both dilutions, this factor, too, was a constant. The only variable, then, in the experiment, was the amount of tumor tissue injected. Therefore, the results show that the lethal effect of a tumor grown in a yolk sac depends to some extent on the amount of tumor tissue inoculated.

3. Pathological Findings in Chicks with Serially Transferred Tumors

Gross observations.—These were first made on chicks that died from yolk sac tumors. Postmortem degeneration was often sufficiently advanced to have caused changes, so for most of our gross observations we relied on living chicks from eggs that had been incubated 13 or 14 days and which had been inoculated with ET, EAT 1, or EAT 2 after the tumors had developed increased lethal properties. These chicks were generally smaller than those from non-inoculated eggs incubated for the same time. Petechiae and congested blood vessels were evident in some. Also, in contrast to the control chicks, the livers of the tumor-bearers were apparent through their stretched, translucent abdominal walls.

On opening the tumor-bearing chicks, their livers were found to be enlarged. The color of these livers varied considerably, presenting 3 main types of surface coloration—either a pale or red or partly pale and partly red. Some livers revealed very deep red blotches. The edges and tips of the lobes of all three general types were frequently yellow or gray-yellow, and occasionally showed pin points of red. Some livers, in addition to manifesting yellow or gray-yellow margins, showed patches and streaks of the same color over their surfaces.

The hearts of tumor-bearing chicks were larger than those of the controls. When squeezed with forceps they were much softer and flabbier than normal, with large, red atria.

The developing kidneys of tumor-bearers were only occasionally of a deeper pink than in the controls.

The blood of tumor-bearing chicks was more

watery, and the blood vessels of the membranes were much paler than those of controls.

Microscopic observations.—These were made principally on the tissues and organs of chicks of the type relied on for gross observations.

In the liver the earliest and most consistent abnormality seen was a wide separation of its tubular cords (Figs. 3, lower and 4). Since the reticuloendothelium was adherent to the separated cords, the lesion represented a sinusoidal dilatation rather than an edema. When the condition was general throughout the liver the sinusoids were widely dilated, but when the lesion had a patchy distribution the degree of dilatation was usually not so great. Some dilated sinusoids contained relatively few erythrocytes, in which instance the liver was pale on gross appearance. But others contained more; indeed, some were packed with erythrocytes (Fig. 5). When the sinusoids were well filled with erythrocytes the liver was red upon our gross examination. In some instances large pools of erythrocytes with no liver cords in their midst were seen; these accounted for the deep red blotches occasionally noticed on the surface. Globules of muddy pigment were seen both in reticuloendothelial cells and free in the sinusoids.

In many of the livers, areas near the surface were seen in which sinusoids were packed with erythrocytes that had lost their cytoplasm and whose nuclei showed pyknosis and fragmentation (top of Fig. 4 and Fig. 6). Where the degenerative changes in erythrocytes were not advanced, the adjacent liver parenchyma was healthy; but when the changes in erythrocytes were advanced, the adjacent parenchyma showed degeneration or necrosis. The early change was a separation of the individual cells of the cords, (Fig. 6), the late, a failure of staining (top of Fig. 3). Sometimes, particularly in the older livers, calcium deposits were seen in the necrotic parenchyma. On gross observation, the necrotic areas appeared yellow.

Although the sinusoids containing degenerating or necrotic erythrocytes were usually well filled with them, they were not generally nearly so dilated as the sinusoids in the remainder of the liver. The condition illustrated in Fig. 4, in which the sinusoids are so distended with degenerating and necrotic erythrocytes as to be evident in a low power photograph, is the exception rather than the rule.

In no instance were emboli of tumor cells, or any lesion that resembled a secondary tumor, seen in the liver.

Microscopic investigation revealed no abnormality in the heart muscle or in most of the developing kidneys. In some kidneys, however, there was congestion or even hemorrhage.

In serial sections cut from skin and subcutaneous tissue demonstrating small hemorrhages the extravasated erythrocytes were usually associated with a developing artery and vein. In some instances the wall of the developing vein appeared deficient enough to allow the escape of erythrocytes into the surrounding soft mesenchyme. Many hemorrhages were distributed along the developing plexus between dermis and subcutaneous tissue.

Blood findings.—Blood studies were made on chicks from 13 and 14 day living eggs selected at random that had been routinely inoculated with tumor ET between its 25th and 30th transfers, and on chicks from 16 and 17 day living eggs, also selected at random, that had been routinely inoculated with tumor ECT between its second and fifth transfers. The weights of the tumors contained in the yolk sacs of those chicks, from which blood studies were made, were ascertained. For control studies, noninoculated eggs incubated for the same period were used.

The hemoglobin levels of the 13 and 14 day chicks, whose yolk sacs contained tumors of numerous egg transfers in eggs thereby gaining enhanced lethal properties, are presented in Fig. 7, together with the weights of the tumors recovered. The hemoglobin levels of the 16 and 17 day chicks, whose yolk sacs contained tumors of only a few egg passages and thus had not developed enhanced lethal properties are presented in Fig. 8, together with the weights of the tumor recovered. In neither graph is a positive correlation between tumor weight and the degree of anemia of the chick evident. A comparison of the two graphs, however, reveals that more chicks developed anemia and that it was more severe when the tumor had been transferred many times in eggs.

Although photographs made of the blood of many normal chicks incubated for 13 or 14 days contained a considerable number of erythroblasts, those made from the blood of tumor-bearing anemic chicks of the same age contained, in general, more of them. Furthermore, many of these were of a younger type, and megaloblasts were not uncommon (Fig. 9). The cytoplasm of the erythroblasts in the blood of chicks with severe anemia was extremely deficient in acidophilic material; hence these cells often appeared as blue-tinted ghosts. The younger erythrocytes also were extremely pale. Indeed, old erythrocytes were the only type seen that were well filled with hemoglobin, and not all these were. Large numbers of degenerating cells of the erythrocyte series were also present. Degenerating erythrocytes, how-
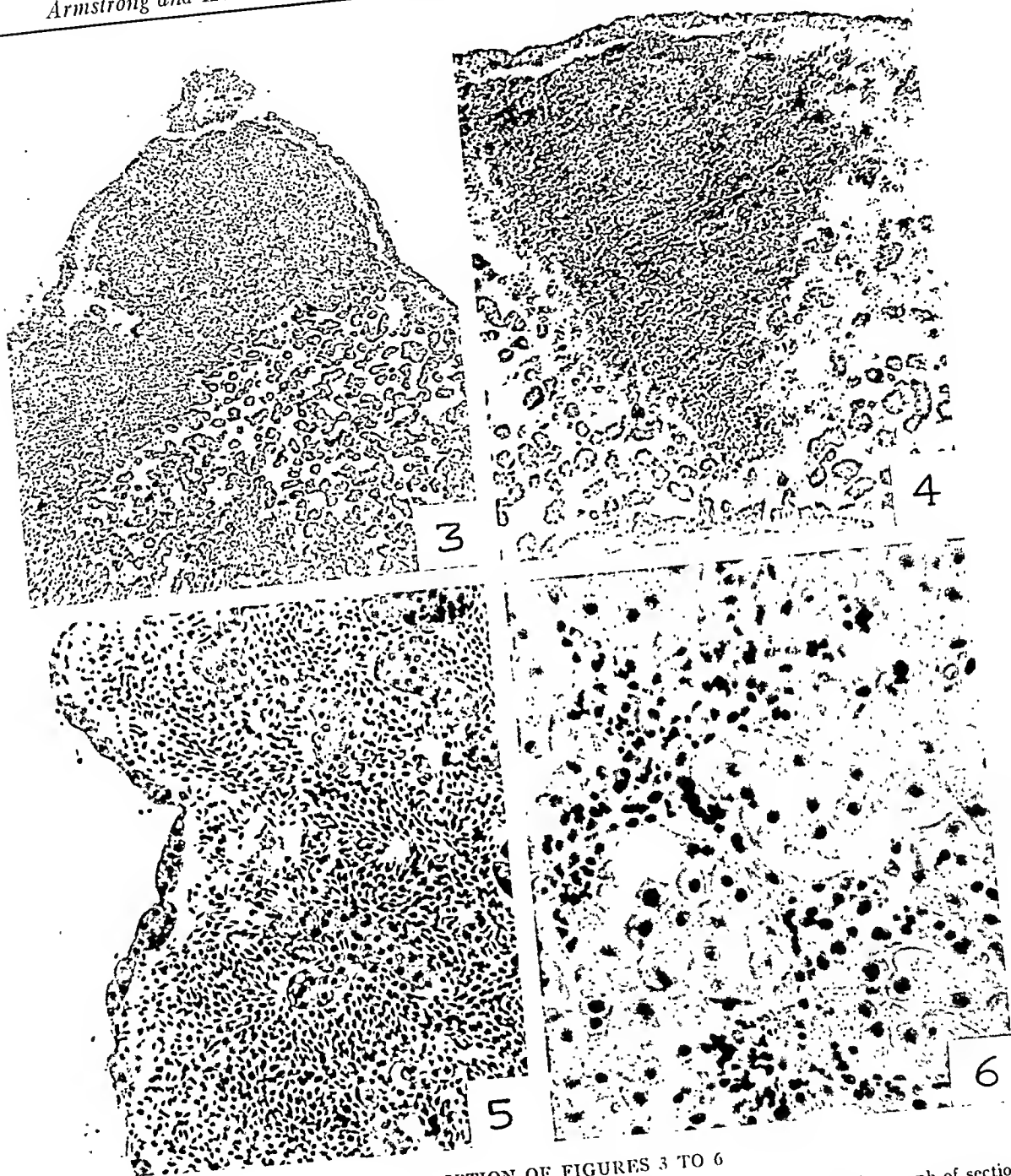
Fig. 3.—Low-power photomicrograph of section of liver showing marginal necrosis above and dilated sinusoids below. Mag. × 60.

Fig. 4.—Low-power photomicrograph of section of liver showing triangular area of early necrosis containing sinusoids packed with degenerating erythrocytes. Outside this area the sinusoids are widely dilated. Mag. × 100.

Fig. 5.—Medium-power photomicrograph of section of liver showing part of pool of erythrocytes near surface with only a few liver cords within it. Mag. × 200.

Fig. 6.—High-power photomicrograph of section of liver taken through area where parenchymal degeneration is beginning. The sinusoids in this area are packed with degenerating erythrocytes and cells of the parenchymal cords are separating from one another. Mag. × 500.
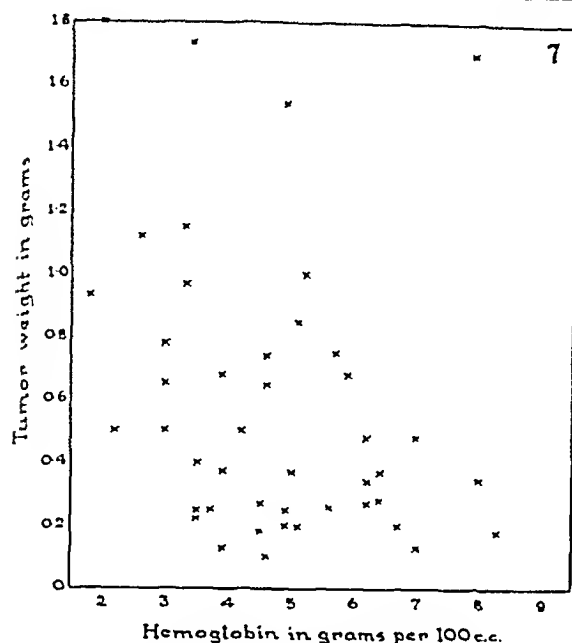
FIG. 7. —Hemoglobin values and tumor weights in 13 and 14 day chicks selected at random whose yolk sacs contained C3H mouse mammary tumors (ET) that had undergone from 25 to 30 transfers in eggs. The hemoglobin value of normal chicks of this age was 7.93 ± .08.
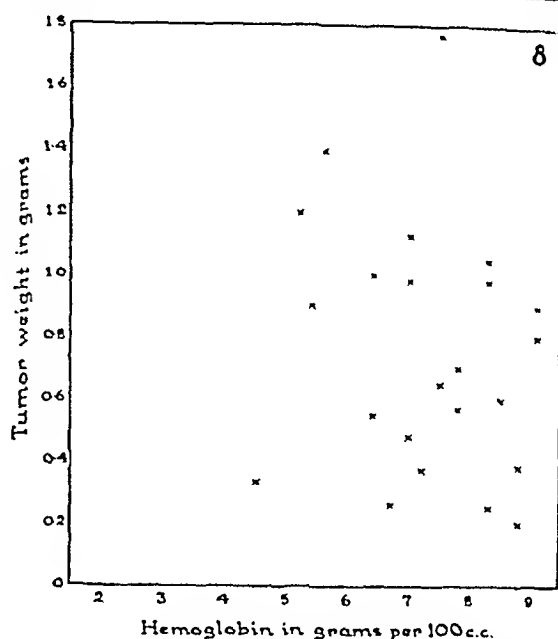
FIG. 8.—Hemoglobin values and tumor weights in 16 and 17 day chicks selected at random whose yolk sacs contained C3H mouse mammary tumors (ECT) that had undergone from 2 to 5 transfers in eggs. Hemoglobin value of normal chicks of this age was 8.86 ± .21.

ever, were seen in the blood films of normal chicks of this age, but in general they were not so numerous. Only a few granular leukocytes were seen in the films. Lymphocytes are not present in the blood at this stage of development; Sugiyama (12) states that they do not appear until the 17th day. Some thrombocyte counts were made by the indirect method on the blood of both normal and tumor-bearing chicks, the lowest being 1,000, and the highest 24,000 per cu. mm. of blood. Usually the count was somewhat higher in tumor-bearing, anemic chicks than in normal chicks.

Blood sugars.—Preliminary work only has been done on this phase of the investigation. The blood sugar of 8 noninoculated chicks incubated for 14 days ranged from 115 to 140 mgm. per cent. The blood sugar of 8 chicks inoculated with ET at approximately its 40th transfer ranged on the 14th day of incubation from 19 to 108 mgm. per cent but 5 of the 8 chicks had blood sugar levels of 27 mgm. per cent or lower.

DISCUSSION

On whether the continued serial Transfer of mouse tumors in yolk sacs is associated with an increased lethal effect on chicks.—Heilman and Bittner (7) working with mammary tumors from C3H and A strain mice, first described such an effect. Hungate and Snider (8), however, found "no significant

increase in the toxicity" of a dba mouse mammary carcinoma that was transferred in eggs 32 times. Their method of inoculation differed from that of Heilman and Bittner and from ours in that the tumor tissue was washed in Tyrode's solution. Less tumor tissue was injected, the extra embryonic coelome route was used for inoculation, and the tumor tissue for serial transfer was selected from the longer lived eggs. Since our dilution experiment showed that less tumor tissue with the same amount of a mixture of fluids from the chicks decreased the lethal effect of the tumor developing on yolk sac inoculation, our results suggest that the failure of Hungate and Snider to observe the same degree of lethal effect seen by Heilman and Bittner and by us, was due more to the small amount of tumor tissue they injected than it was to their washing the tumor tissue in Tyrode's solution to free it from the fluids of the chick. They suggest that "under different experimental conditions an increase in toxicity might result," and indeed in their discussion they note having occasionally observed instances of this phenomenon.

Our findings confirm those of Heilman and Bittner (7). Our experience with the C3H mouse mammary carcinoma seems to have been very much like theirs. We did not, however, observe the very rapid development (on the first few trans-

fers) of an increased lethal effect on cultivating A strain tumors that they did. In our hands two A strain carcinomas developed increased lethal qualities only after 8 and 20 transfers respectively.

We conclude, then, that the serial transfer of mouse mammary tumors in eggs is associated with the development, after a varying number of transfers, of an increased lethal effect on chicks. Moreover our dilution experiment indicates that the increased lethal effect is associated with the tumor cells rather than with the fluids of the chick.

On where and how the lethal effect is exerted.— Our results suggest that the cause of death of chicks bearing tumors with increased lethal properties is to be found in their blood, hearts and livers. The relationship of these lesions to one another will now be discussed.

Taylor, McAfee and Taylor (15) were the first to observe liver lesions in chicks with yolk sac tumors, noting that this organ was pale and blotchy. Kynette, Taylor and Thompson (10) extended these studies and observed abnormal accumulations of erythrocytes, erythrocyte desposits, open frameworks of liver cells separated by spaces, and areas of necrosis in the livers that they examined.

FIG. 9.—Oil-immersion photomicrograph of blood film of chick with severe hypochromic anemia resulting from yolk sac tumor of many egg passages, showing 2 erythrocytes well filled with hemoglobin(ery.w.f.), 3 erythrocytes containing scarcely any hemoglobin(ery.p.f.), 3 erythroblasts with very little hemoglobin(erb.) and a megaloblast(meg.). Mag. × 1200.

Our observations are in agreement with theirs in these respects. We did not, however, observe any abnormal proliferation of cells bordering sinusoids, a point they stressed.

Twombly and Meisel (17) have also studied the lesions in livers of chicks with yolk sac tumors. They noted that the organ often showed areas of widespread necrosis, but that in less damaged areas "one is apt to come upon areas of blue-staining cells lining the sinusoids." It is their impression that these are cancer cells growing in a nonreacting host without stroma and that their presence accounts for much of the mortality of tumor-inoculated eggs.

Our histological findings offer no support for Twombly and Meisel's thesis. Although they obtained tumor growths in mice inoculated with liver tissue obtained from tumor-bearing chicks, and therefore reasoned that the liver must contain viable tumor cells, we found no histological evidence indicating that the liver lesion is primarily due to either emboli of tumor cells or secondary growths. Liver damage, we think, is to be otherwise explained.

It is known that severe anemia can result in cardiac dilatation and congestive heart failure (18). Since the hearts of the anemic chicks were soft and flabby, it may be assumed that they were affected by the anemia. If the hearts were affected sufficiently to become incompetent, the sinusoidal dilatation observed in the livers of the anemic chicks could be considered to be the result of a congested state induced by the heart's incompetence. In strong support of this contention we found that the extent and degree of dilatation of liver sinusoids bore a rough but generally consistent relationship to the degree of anemia exhibited by the chick. Hence, we conclude that anemia is the cause of an incompetent heart and it in turn is the cause of congested and dilated liver sinusoids.

It might be thought that the degree of hypoglycemia indicated by our preliminary experiments might be a factor in causing the death of the chicks. There is evidence, however, that a degree of hypoglycemia that would cause convulsions in an adult has singularly little effect on the embryo and even on the newborn. Zondek and Wolfsohn (19) have reported finding low blood sugar levels in newborn infants, in some instances obtaining a value of 0 mgm. per cent. In work performed in our own laboratory, as yet unreported, we have found the newborn rabbit to have an extremely low blood sugar level, comparable with those in the tumor-bearing chicks. It does not seem likely, therefore, that the hypoglycemia
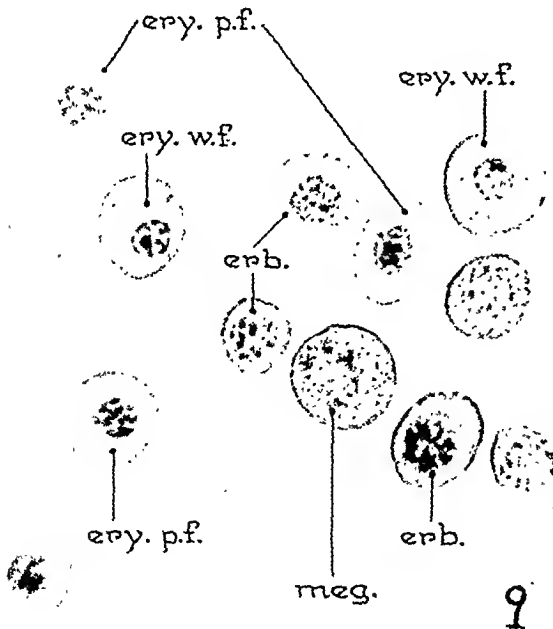
of the tumorous chicks would necessarily be a direct cause of their deaths. But nevertheless a sustained hypoglycemia, by making less sugar available for glycolysis in the myocardium, might well assist the anemia in making the heart incompetent.

The degeneration and necrosis of liver parenchyma is not so easy to explain as the dilated sinusoids. Before attempting to do this it is helpful to point out that this lesion occurs when the liver is in a state of active development. Tubular cords of parenchyma are continually budding, growing, and uniting with one another to establish the pattern of the sinusoidal spaces between them. Even under normal conditions it appears that this process achieves only with difficulty an efficient circulatory pathway through the peripheral part of the organ, because small areas of degeneration and necrosis, identical with the larger ones seen in the anemic chicks, were found in this region in some of our controls. With anemia and a less competent heart, it is only to be expected that the oxygenation of the peripheral parts of the liver would be correspondingly less adequate and hence that more degeneration and necrosis would result. It could be expected also that stagnant circulation in these peripheral areas, before they actually become necrotic, could account for the aggregations of erythrocytes seen in the sinusoids either in or about the degenerating or necrotic parenchyma.

We conclude, then, from our studies that anemia is the basic pathological lesion produced by a yolk sac tumor and that the other lesions seen in the chick are consequent to it. We have also shown that the severity of the anemia produced by a yolk sac tumor is much greater after a tumor has been transferred many times than it is when a tumor has been transferred only a few times (compare Figs. 7 and 8). Hence, we conclude further that the increased lethal effect observed when mouse tumors are cultivated serially in eggs represents an increased anematizing effect.

On the nature of the anematizing effect of tumors.— That a malignant growth tends to cause a hypochromic anemia in its host has long been appreciated. Different explanations have been offered for this phenomenon at different stages in the development of knowledge about cancer. These will now be briefly considered in the light of our findings.

First, the older views may be grouped together since they postulate that tumors cause anemia indirectly. They ascribe the anemia variously to hemorrhage, bacterial infection, necrosis of tissue with the absorption of decomposition products, an impaired food intake of the host, or to other conditions brought about by the presence of the tumor. Our finding of severe anemias in chicks with small tumors that evidenced little hemorrhage or necrosis and no bacterial infection does not support the idea that the anemia of malignancy is only an indirect effect of the malignancy.

Second, Taylor and Pollack (16) have suggested that a much closer relationship between malignant growths and anemia exists than has been hitherto suspected. On finding anemia in mice with very early implants and in mice treated with carcinogens even before malignancy developed, they suggested that a cancerous growth or a precancerous condition might have a direct and destructive effect on hemoglobin or inhibit its production. Later Taylor, McAfee and Taylor (15) on finding significant degrees of anemia in chicks with small mouse mammary tumors growing in their yolk sacs stated "it may be presumed that the tumor cells produce a substance or substances which when liberated into the blood stream exert in some manner an inhibitory influence upon the blood hemoglobin concentration." In the same study they found this effect to vary directly with the size of the tumor. In further work with mice Taylor (13) found further evidence to indicate that the anemia varied in relation to the size of the tumor and suggested that each tumor cell makes its contribution toward producing anemia.

Although the theory suggested by Taylor and his co-workers represents in our opinion a very important advance, we do not find it adequate to explain our findings. The concept of a direct effect varying in relation to tumor size does not explain why we should have observed more severe anemias with yolk sac tumors of many serial passages that we did even with larger tumors in their early egg passages. It should be noted in this respect that our study differed from that of Taylor, McAfee and Taylor (15) in that we studied the anemia produced by serially transferred tumors, whereas they studied that produced by first generation egg tumors derived from a standardized transplanted tumor.

Third, Dunning and Reich (2) observed the degree of anemia in the hosts of induced and transplanted sarcomas to be related to the malignancy of the growth. Taylor (13), moreover, also brings this concept into his theory by suggesting that the rate of decrease in hemoglobin concentration with increasing tumor size is related to the degree of malignancy inherent in the tumor concerned.

In order to examine the concept of the anematiz-

ing effect of a tumor being related to its degree of malignancy in the light of our findings, it is necessary to point out first that what we term the anematizing effect probably has functions other than causing anemia. Taylor and Pollack (16) were first to suggest this. Noting that Craig, Bassett and Salter (1) have shown that the cytochrome content of tumor tissue is lowered, and that Greenstein, Jenrette and White (5) demonstrate that the introduction of malignant tissue into an animal greatly decreases liver catalase, and that cytochrome, liver catalase and hemoglobin all have the same hemin prosthetic group, they suggested that an effect on all 3 porphyrin compounds was exerted in the same manner. Greenstein (6) also points out the relationship between liver catalase and hemoglobin and has suggested a tumor activity directed against the synthesis of the hematoporphyrin prosthetic group. Greenstein and co-workers (3, 4) furthermore make the very important observation that the liver catalase lowering effect of tumors is not an effect of rapidly growing cells, embryonic cells, or a combination of the two, for they have shown that the catalase of the liver is not reduced in regenerating livers, in the livers of pregnant animals, or in animals with growing implants of embryonic tissue. It would seem, therefore, that the effect exerted against these porphyrin compounds is not to be related to the physiological properties of rapidly growing or undifferentiated cells; indeed in our work tumors were grown in an embryonic host that abounded in embryonic and rapidly growing cells, yet anemia did not develop unless tumor tissue also was growing in this host. Hence a rapid growth and an undifferentiated state of cells (the chief criteria for estimating a high degree of malignancy in tumors) are not factors which in themselves produce anemia.

That we observed the anematizing effect to become enhanced without a corresponding increase in tumor size suggests to us that a qualitative rather than a quantitative change occurred in it after tumors had been transferred many times in eggs. Since we worked with tumors from mice known to carry the milk influence, and since many viruses have been shown to attain increased virulence on their serial passage in eggs, we question if our results do not suggest that the anematizing effect is not so much in the nature of a true cellular secretion or excretion as it is the property of some agent associated with, and perhaps causing, the tumor process, and of a kind that might be expected to exhibit variation on prolonged serial transfer in eggs.

SUMMARY

The serial cultivation of mammary tumors from C3H and A strain mice in the yolk sacs of hens' eggs resulted in chicks dying in increasing numbers and progressively earlier. The lethal effect of any tumor developing from the inoculation of eggs with tissue from tumors that had been transferred often enough to have gained enhanced lethal properties, was found to depend to some extent on the amount of tumor tissue with which the yolk sacs were inoculated.

The chief pathological findings in the chicks so affected were: hypochromic anemia, enlarged flabby hearts, and enlarged livers, often showing peripheral necrosis. Reasons are given to show that the basic lesion produced by the tumor in the chick is hypochromic anemia and that the other pathological lesions are consequent to it. In view of this and of the fact that the severity of anemia produced by the tumors increased with their serial transfer in eggs, it was concluded that the increased lethal effect of tumors that develops on their serial egg transfer represents an increased anematizing effect.

Since the anematizing effect on the yolk sac tumors we studied was not found to be related to their size, and since they were mammary tumors from strains of mice that carry the milk influence, and since the effect became enhanced on the serial transfer of the tumors, the suggestion is made that the anematizing effect may be more in the nature of some agent associated with the tumor process than a secretion or excretion of the tumor cell proper.

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The Use of the Mouse Eye in Transplantation Experiments*

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The anterior chamber of the eye has proven an almost ideal site for tissue transplantation. The technic of transfer is simple, a high percentage of takes is obtained and the growing tissue can be followed by direct visual observation or even subjected to microscopic examination. A further advantage derives from the fact that the chamber supports the growth of heterologous tissues whereas, in other bodily sites, such tissues invariably fail to survive. Moreover, tissues grown in the chamber are usually readily separable from the tissues of the host; they contain little desmoplastic reaction, and in general are excellent material for chemical or immunological study.

In this laboratory, animals such as the guinea pig and rabbit have been used extensively in experiments on transplantation of organs and tissues to the anterior chamber of the eye. The expense of securing and maintaining such animals is considerable in relation to that entailed in obtaining and providing for mice and for this reason, a modification of the technic has been perfected to permit the easy and rapid utilization of mice for the same purpose.

METHOD

The technic used for anterior chamber transfer in larger animals is unsatisfactory when applied to mice. Securing the mouse to an animal board is difficult and time-consuming. A local anesthetic is inadvisable because of the proximity of the conscious animal's teeth to the operative field. The combination of a small eye, a resentful animal and a cumbersome restraining apparatus makes an ordeal of the procedure.

The technic adopted eliminates such difficulties. General anesthesia is effected by suspending the mouse by its tail in a drinking tumbler containing several gauze sponges soaked in ether. Sufficient anesthesia is obtained in 30 seconds, and as this step is carried out by an assistant, the time may be utilized by the operator in placing the tissue to be transferred in a trocar. The trocar is made by

shortening the bevel at the tip of a 20-gauge hypodermic needle. A suitable, tight-fitting plunger can be manufactured or obtained simply by selecting a wire stylet of proper size from the stock supplied with the needles and applying a knob of sealing wax or other plastic material to one end.

A minute fragment of tissue is placed in the mouth of the trocar and manipulated into its barrel. This is usually readily accomplished by retracting the plunger to exert suction and prodding the fragment with a fine needle. Sometimes this operation may be irksome, particularly if the tissue is dry or sticky, but caution should be urged against the use of so-called physiological saline in attempt to overcome the difficulty. Stock saline solutions in general use are not physiological and are often sufficiently toxic to cause death of the tissue.

The anesthesia rarely lasts longer than a minute and subsequent procedures must be executed rapidly. The mouse is held loosely in the left hand and the lids of its right eye forced apart with the thumb and index finger. Slight pressure with these fingers causes the eye to protrude sufficiently to allow adequate exposure for the operation. The anterior chamber is opened close to the upper border of the corneo-scleral junction by means of a short, quick jab with a double-edged corneal knife. The knife is of a size generally used in ophthalmological work and is readily secured in any instrument house. Single-edged knives or pointed Bard-Parker blades are not satisfactory for they result in a triangular incision through which the iris may herniate. The incision is made only of sufficient length to admit the trocar and must be accomplished entirely by the downward thrust of the knife for the instability of the protruding eye prohibits side cutting. The point of the knife should be directed slightly forward in making the incision in order to enter the chamber without cutting the iris.

The trocar, held between the thumb and middle finger of the right hand, is inserted into the chamber through the incision and the fragment expressed by pushing the plunger with the index finger. In order to prevent extrusion and escape of the fragment through the excision, all pressure exerted on

the animal by the left hand should be released before withdrawal of the trocar. The fragment now free in the chamber, is forced into a wedged position at the inferior angle of the iris by applying light pressure along the corneal surface with a blunt instrument. The incision is not closed.

Considerable trouble may be encountered in preventing the escape of soft, slippery tissues such as embryonic brain when the trocar is withdrawn. This difficulty may be circumvented by incising the iris as well as the cornea at the limbus and directing the trocar behind the superior half of the iris, through the pupil and into the inferior portion of the anterior chamber. With withdrawal of the trocar, the fragment is almost invariably caught at the pupillary border and retained in the chamber.

The whole operation can be performed rapidly after short practice. The average speed in this laboratory is 2 mice a minute and further acceleration may be obtained if 2 anesthetists are employed. However, it should be emphasized that sterility is essential; instruments should be boiled and aseptic technic maintained throughout the procedure. The mouse eye is apparently much more susceptible to infection than is the eye of the guinea pig or rabbit.

RESULTS

The mouse eye has been used extensively in this laboratory for the past 3 or 4 years, particularly in the homologous and heterologous transplantation of cancer and embryonic tissue. The results have been most satisfactory in experiments in which only small growths of tissue were desired. The anterior chamber of the mouse eye is not large enough to contain growths of the size needed for
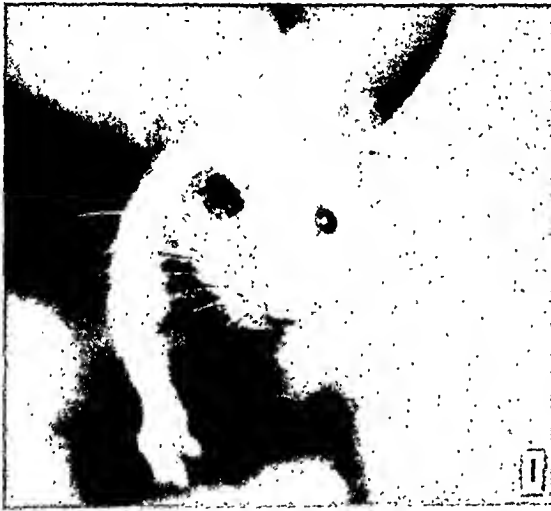
FIG. 1.—Mouse of strain A bearing anterior chamber transplant of embryonic mouse tongue treated with methylcholanthrene. This photograph was taken 83 days after transfer. Histologically, the tumor was an epidermoid carcinoma.

FIG. 2.—Mouse of C3H strain bearing anterior chamber transplant of Brown-Pearce rabbit tumor. This photograph was taken 140 days after transfer and represents a recurrence of growth after partial regression of transplant. Histological sections at death were identical with those of same tumor grown in rabbit.

DESCRIPTION OF FIGURES 3 TO 8

FIG. 3.—Section of eye of C57 mouse bearing a transplant of a mouse ovarian embryoma. The animal was killed 2½ weeks after transfer. Mag. × 35.

FIG. 4.—Higher power view of previous section. Mag. × 320.

FIG. 5.—Section of eye of Swiss mouse bearing transplant of mammary carcinoma originating in CBA mouse. Attempts to transfer tumor from CBA mouse to subcutaneous tissues of Swiss mice were unsuccessful but high percentage of takes was obtained when tumor tissue from

Swiss eyes was transferred to subcutaneous spaces of other Swiss mice. Mag. × 50.

FIG. 6.—Higher power view of previous section. Mag. × 350.

FIG. 7.—Section of eye of Swiss mouse bearing a transplant of Cloudman melanoma. As in previous case, transfer from the eye to subcutaneous space resulted in takes whereas direct subcutaneous transfer from parent strain to Swiss was unsuccessful. Mag. × 50.

FIG. 8.—Higher power view of previous section. Mag. × 225.
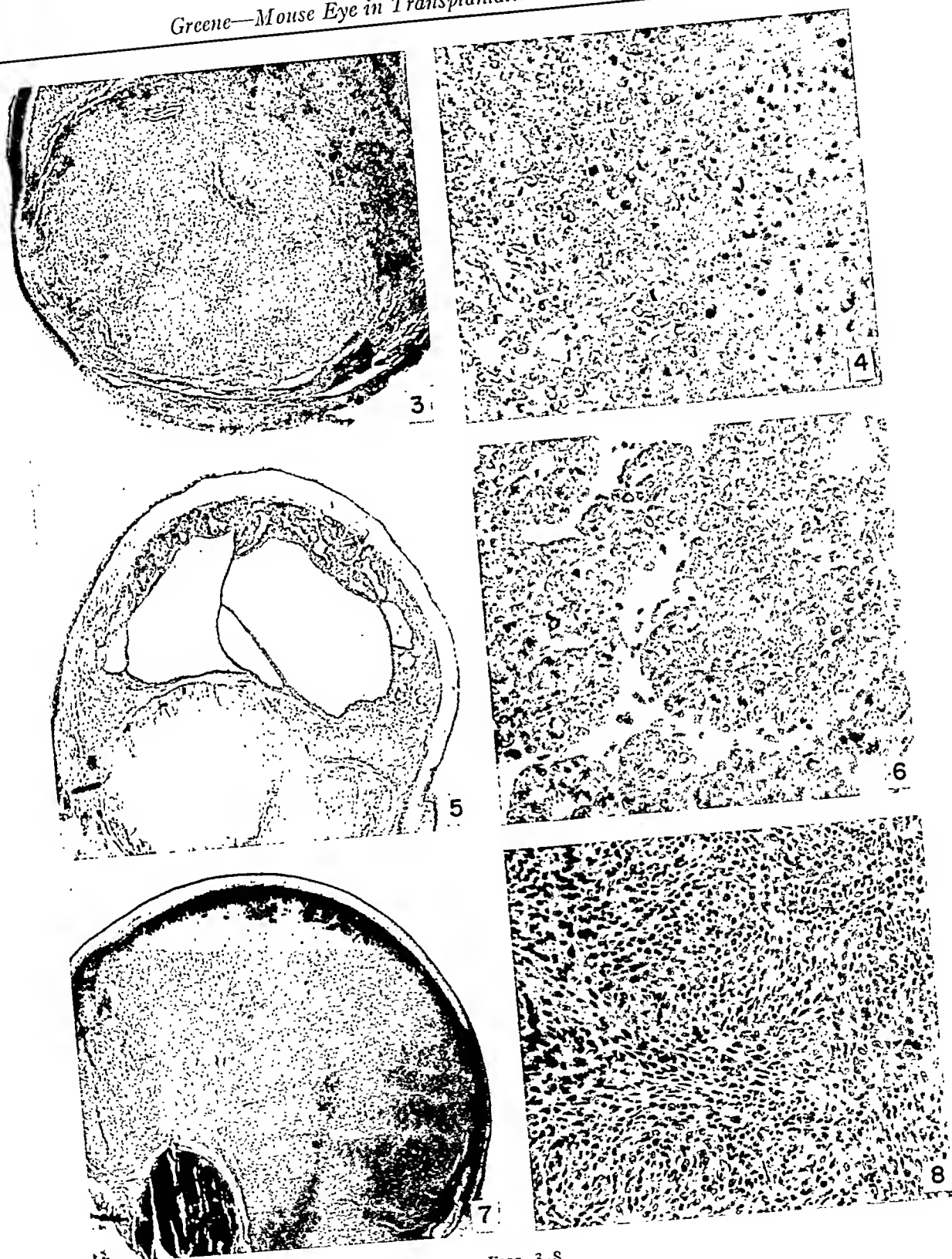
chemical or immunological studies and other species are more suitable for such purposes. Large growths do occur in the mouse eye but they are necessarily associated with rupture of the cornea and external protrusion. Infection is always present in such cases and the tissue is valueless for further passage or other experimentation.

Homologous transfers.—The transfer of mouse tumors to the anterior chambers of eyes of other mice results in a high percentage of takes and rapid growth. The age or sex of the recipient has played no observable part in the behavior of the graft in our series of transfers. Moreover, the so-called influence of strain appears to be largely negated when the anterior chamber is used.

Reports in the literature suggest that many mouse tumors are strain-specific. This has not been the case with the great majority studied in this laboratory. It is true that, in early stages of development, mouse tumors, like tumors in other animal species, are dependent in nature and are transplantable only autologously or to other animals of the same strain. But, with continued development in the original host, they attain autonomy and the ability to survive and to grow on homologous and heterologous transfer. Thus, only autologous takes or takes in animals of the same strain may result from the transfer of tissue obtained at biopsy whereas growth in foreign strains and even in alien species occurs with material derived from autopsy, 1 to 3 months later. It is suggested that the standard strain-specific mouse tumors, well known in all cancer research laboratories, were originally transferred from a spontaneous growth before autonomy had been attained.

In any case, it has been our experience that, if the primary host survives a sufficient period of time after the origin of the tumor, the growth loses its "strain specificity" and becomes transplantable in many strains. At the same time a variation persists in the ease with which the autonomous tumor is transferred to various strains. Thus, one tumor originating in a C3H mouse could be trans-ferred directly to dba mice but not to C57 mice. On the other hand, when the same C3H tumor grown in a dba mouse, was used, a high percentage of takes occurred in C57 mice. Such results suggested that the strain of the donor exerted an influence on the outcome of transfer and it was felt, for many reasons, that this might be referable to an antagonistic interaction between the connective tissues of the intended recipient and the tumor stroma carried along with the parenchyma at transfer (1).

The point to be emphasized in the present connection bears on this suggestion, for takes occur on direct transfer from the primary host to the eye of the new strain in cases in which successful subcutaneous transplantation requires the intermediation of another host. After growth in the eye, the tumor can be readily transferred to the subcutaneous space of the new strain and carried by serial passage in that site. If, as suggested, incompatibility reactions between the connective tissues of the donor and recipient form the basis of the failure of primary subcutaneous transfer, then it must be assumed that such reactions do not occur in the anterior chamber. Other experiments substantiate this assumption and offer a plausible explanation. Histological study of anterior chamber grafts removed at short intervals after transfer shows that the transplanted stroma dies before vascularization by the new host begins while, in the interim, the parenchyma proliferates in the manner of a tissue culture. Thus, when vascularization eventually occurs and the connective tissues of the new host are brought in contact with the implant, the old stroma has largely disappeared and the basis for any serious interaction has been removed.

Growth is rapid in the anterior chamber so that after several weeks, the cornea ruptures and the tumor protrudes as a fungating mass. Large portions become necrotic, covered with dried crust and may eventually slough off. In such cases, the animal may live long enough for metastasis to occur, but usually the tumor becomes infected

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DESCRIPTION OF FIGURES 9 TO 14

Fig. 9.—Section of eye of C57 mouse bearing transplant of the upper third of 2 mm. C3H embryo. Section was taken 80 days after transfer and shows growth of cartilage and squamous epithelium. Mag. × 50.

Fig. 10.—Section of eye of C57 mouse bearing transplant of intestine from C57 embryo. Section was taken 56 days after transfer. Mag. × 35.

Fig. 11.—Section of eye of Swiss mouse bearing transplant of lung from Swiss embryo. Section was taken at 94 days and shows an epithelialization of alveoli comparable to that seen in so-called lung adenomas of A strain. Mag. × 150.

Fig. 12.—Section of eye of Bagg albino mouse bearing transplant of brain from strain A embryo. Section was taken at 39 days and shows growth of ganglion cells as well as of glial elements. Mag. × 300.

Fig. 13.—Section of eye of strain A mouse bearing a transplant of spleen from strain A embryo. Section taken 390 days after transfer. Mag. × 70.

Fig. 14.—Higher power view of previous section. Note megakaryocytes. Mag. × 500.
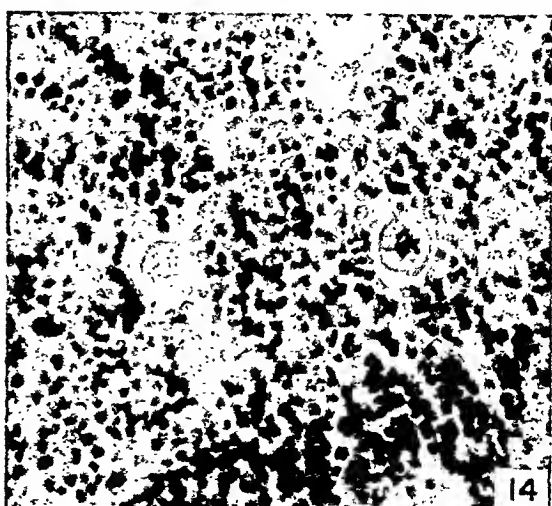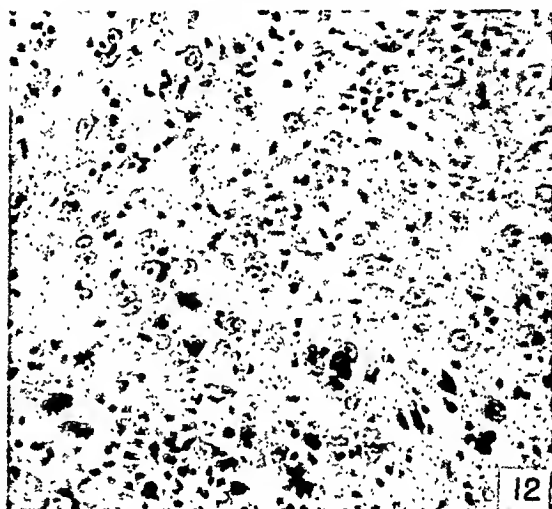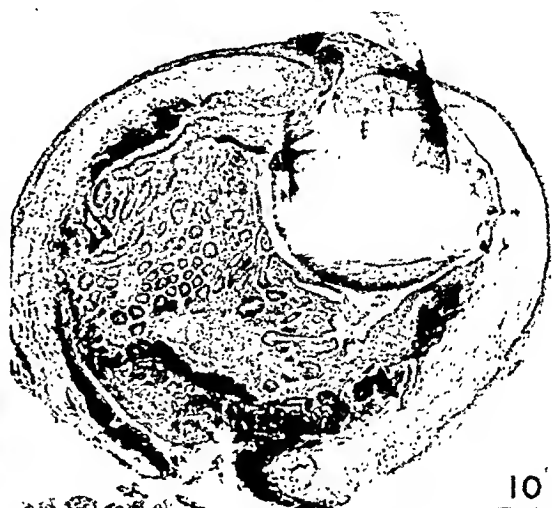
FIGS. 9-14

and death follows without lymphatic extension or dissemination (Figs. 3 to 8).

The transplantation of embryonic tissue is also readily performed in the mouse eye and at the present time, all of the various organs and tissues of the mouse embryo, with the sole exception of the liver, have been successfully transferred to this site. The anterior chamber possesses an advantage in the transfer of small organs as the gonads, spleen and esophagus in that they remain in view and are not lost during early growth phases, as is the case in the expanse of the subcutaneous space. In general, the subcutaneous space is a better nidus for larger organs such as the stomach or lung for it allows their full expansion, whereas in the chamber normal contours and relationships are soon lost and continued growth may lead even to corneal rupture.

A high incidence of takes is obtained and failure appears to depend entirely on infection or faults in technic. The transplanted organ usually reaches its maximum size in from 2 to 3 weeks and no further increase in size occurs. To date, mice bearing organ transplants have been held under observation for as long as 18 months without sign of regression and there is no reason to believe that the grafts will not persist throughout life.

Sections of the transplants obtained several weeks or months after transfer show well developed organs (Figs. 9 to 18). Modifications in differentiation sometimes occur, notably in the lung where squamous metaplasia of bronchial epithelium and epithelialization of alveoli (so-called lung adenomas) are most common.

In several experiments, fragments of adult organs have been used for transfer but potentialities in this direction have not been adequately explored. The fragments survive and actually show some increase in size. Histologically, the structure of the parent organ is duplicated and organization appears normal (Fig. 19). Transplants of adult nerve are an exception in this respect and the aberration is of some interest. The fragments grow rapidly to fill the chamber and, on section, show a disorganized proliferation of Schwann cells bearing a distinct resemblance to tumors derived from these elements (Fig. 20).

The susceptibility of embryonic tissues to the action of carcinogenic chemicals has been reported (2, 3, 5, 6) and the anterior chamber of the mouse eye has been investigated as a nidus for carcinogenesis of this type (Figs. 1, 21 to 24). The technic is essentially identical with that employed in the transplantation of normal embryonic tissues, the only alteration being the addition of a minute crystal of methylcholanthrene to the fragment before transfer. As a rule, an interval of 60 or more days is required before the occurrence of indicative morphological changes, a period of approximately twice the duration required in the subcutaneous space. However, there are distinct advantages, apart from the opportunity for direct visual observation, associated with the use of the eye in such experiments. The eye affords a better medium for the growth of epithelium than does the subcutaneous space, takes occur with higher frequency and structures are reproduced with greater fidelity. Moreover, the connective tissues of the eye are more resistant to the action of carcinogenic chemicals, and sarcomatous growths of the host which might be interpreted as arising in the transplant are far less common.

Heterologous transfers.—Tumors of human and rabbit origin have been successfully transplanted to mouse eyes, although a considerable variation exists in the ease with which the two types of transfer are effected. Transfer from man directly to the mouse gives rise to relatively few takes and much better results are obtained if the tumor is first passed through a guinea pig generation. In contrast, the mouse is a better host for rabbit tumors than is the guinea pig.

The species studied may be classified with respect to the ease of heterotransplantability. A curious relationship was revealed: The mouse and the rabbit constitute one group and man and the guinea pig the other. Transfer within these groups is comparatively easy while transfer between the groups is difficult and attended with a much smaller percentage of takes. It is obvious that

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DESCRIPTION OF FIGURES 15 TO 20

Fig. 15.—Section of eye of Strain A mouse bearing a transplant of kidney from a Strain A embryo. Section taken 28 days after transfer. Mag. $\times$ 35.

Fig. 16.—Higher power view of previous section. Note persistence of embryonic type glomeruli. Mag. $\times$ 300.

Fig. 17.—Section of eye of ZBC mouse bearing a transplant of testicle from C3H embryo. Section was taken 38 days after transfer and shows growth of epididymis as well as of testicle. Mag. $\times$ 30.

Fig. 18.—Higher power view of previous section. Mag $\times$ 225.

Fig. 19.—Section of eye of C3H mouse bearing transplant of ovary from another adult C3H mouse. Mag. $\times$ 85.

Fig. 20.—Section of eye of Swiss mouse bearing a transplant of sciatic nerve from another adult Swiss mouse. Proliferation of cells resembles a neurofibroma of Antoni type. Mag. $\times$ 50.
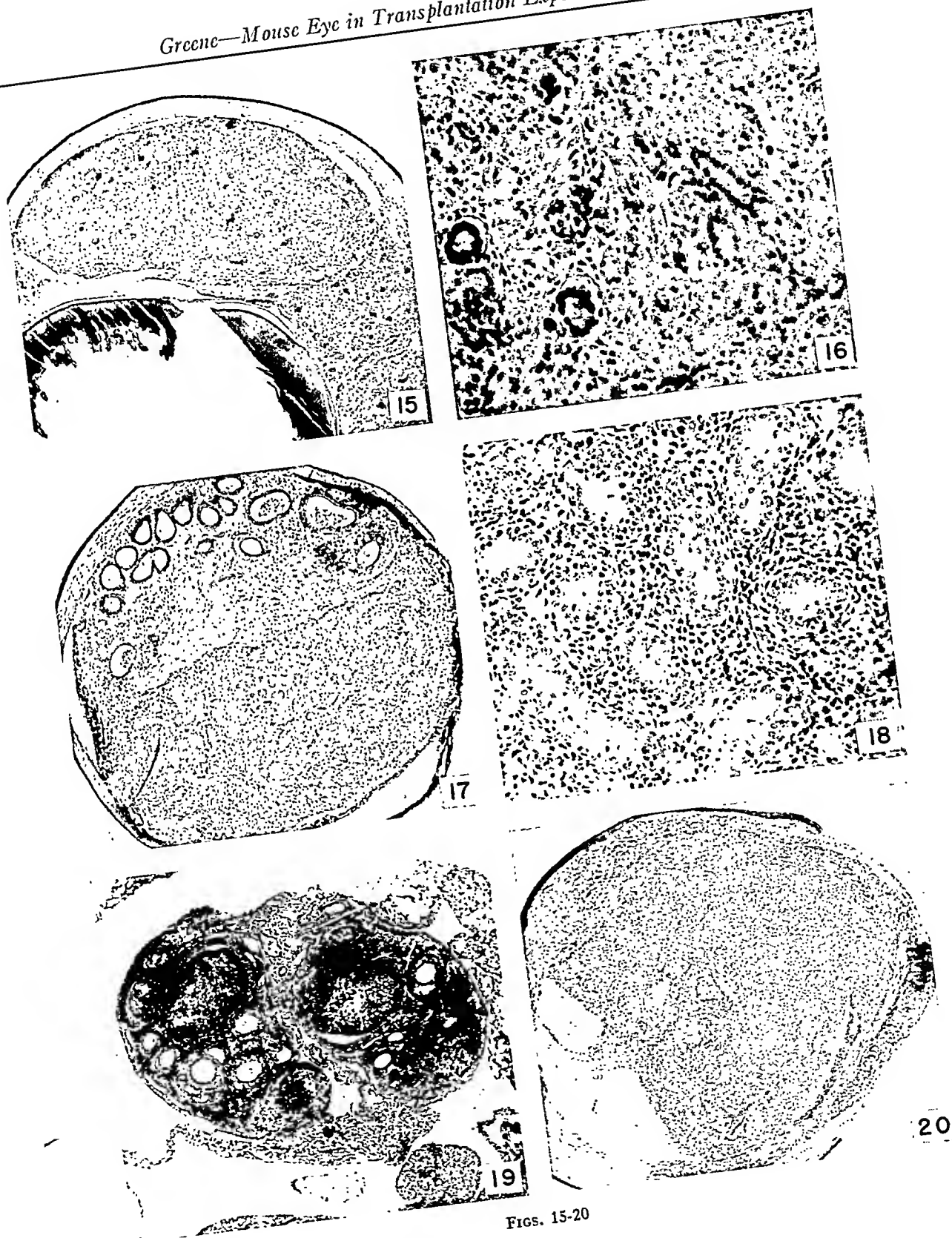
Figs. 15-20

this grouping of species also represents a division with reference to the ability to synthesize vitamin C. The relationship may be purely coincidental but metabolic differences between tumor and host tissues presumably occur and might well account for the observed variations. Pertinent investigations are in progress.

The heterologous tumors most fully studied in the mouse have been a human fibrosarcoma (4) (Figs. 25, 26) and the Brown-Pearce rabbit tumor (Fig. 2). Both give rise to a high percentage of takes, grow to fill the eye and are easily carried by serial transfer if the donor mice are killed before the expanding tumor ruptures the cornea.

The only noteworthy alteration in the growth of the fibrosarcoma in the mouse is a tendency of its cells to round off and assume an epithelioid character in sharp contrast to the obvious fibroblastic nature of the tumor in the guinea pig and in man. Curiously, the same alteration occurs on transfer of this growth to the rabbit. The histological appearance of the Brown-Pearce tumor does not change in the mouse (Figs. 27, 28). A peculiarity of its behavior in this species is the frequency of regression and recurrence. The tumor may grow to form a fungating mass as large as the mouse's head, then undergo regression so complete that no trace of tumor can be found in the atrophied eye. However, recurrence is the rule and in several instances renewed growth was not evident until after the lapse of 6 months.

The mouse eye also affords good growth for the embryonic tissues of other species (Figs. 29 to 32). The same species relationships observed in the heterologous transplantation of tumors holds here but is less pronounced and it is much easier to grow human embryonic tissue in the mouse than it is to grow human cancer. The tissues undergo differentiation and organization and, despite the distinct environmental difference, little variation from normal intrauterine development can be found.

DISCUSSION

The object of the present paper was to point out the potentialities of the mouse·eye as a transplantation site. The experiments cited and the results obtained require further consideration in relation to the special fields to which they pertain but such discussion is not essential to the immediate purposes of this report and will be presented in later papers describing the experiments in more detail. Sufficient evidence has been offered to justify the conclusion that the mouse eye is a good transplantation site offering a better approach to special problems than other bodily regions and deserving more widespread use than is at present accorded.

SUMMARY

A simple technic of anterior chamber transfer in the mouse has been devised. The technic has been successfully applied to the homologous and heterologous transplantation of tumors and of embryonic tissue, the homologous transfer of adult tissues and the production of carcinomas in transplanted embryonic organs. Illustrative experiments are described.

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DESCRIPTION OF FIGURES 21 TO 26

FIG. 21.—Section of eye of strain A mouse bearing a transplant of esophagus from strain A embryo. A crystal of methylcholanthrene was added to esophagus before transfer. Section was taken 69 days after transfer and shows epidermoid carcinoma developing from esophageal mucosa. The excessive keratinization appears to be characteristic of·epidermoid carcinomas of the mouse produced in this manner. Mag. × 35.

FIG. 22.—Higher power view of previous section. Mag. × 225.

FIG. 23.—Section of eye of C3H mouse bearing a transplant of forestomach from a strain A embryo. A crystal of methylcholanthrene was added to the fragment *before* transfer and the animal was killed 55 days afterwards.

The stomach with its lumen filled with keratin occupies the expanded anterior chamber and small epidermoid carcinoma is arising at one pole. Mag. × 50.

FIG. 24.—Section of eye of C3H mouse bearing transplant of forestomach from a ZBC embryo. A crystal of methylcholanthrene was added to the fragment before transfer and animal was killed 98 days afterwards. Section shows an invading epidermoid carcinoma with much less keratinization than is usually observed. Mag. × 85.

FIG. 25.—Section of eye of C57 mouse bearing a transplant of a human fibrosarcoma. Mag. × 50.

FIG. 26.—Higher power view of previous section. Mag. × 300.

FIGS. 21-26

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DESCRIPTION OF FIGURES 27 TO 32

Fig. 27.—Section of eye of strain A mouse bearing transplant of Brown-Pearce rabbit tumor. Animal was killed 10 days after transfer. Mag. × 60.

Fig. 28.—Higher power view of previous section. Mag. × 300.

Fig. 29.—Section of eye of Swiss mouse bearing transplant of embryonic human lung. The animal was killed 40 days after transfer. Mag. × 60.

Fig. 30.—Higher power view of previous section to show contiguous bronchial buds. Mag. × 500.

Fig. 31.—Section of eye of Bagg albino mouse bearing a transplant of kidney from guinea pig embryo. Animal was killed 43 days after transfer. Mag. × 50.

Fig. 32.—Higher power view of previous section. Mag. × 225.
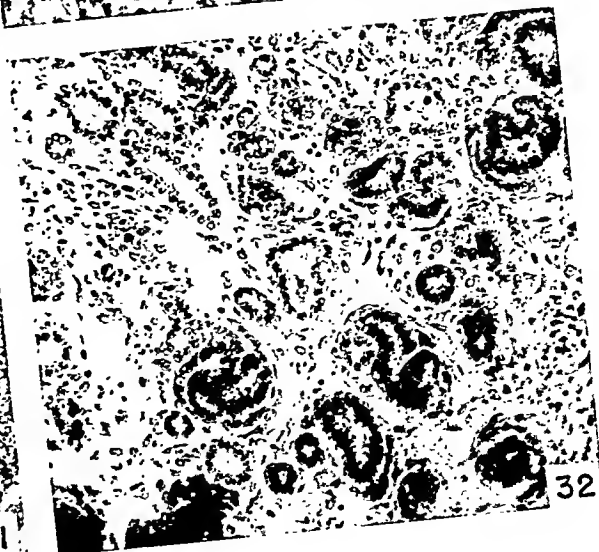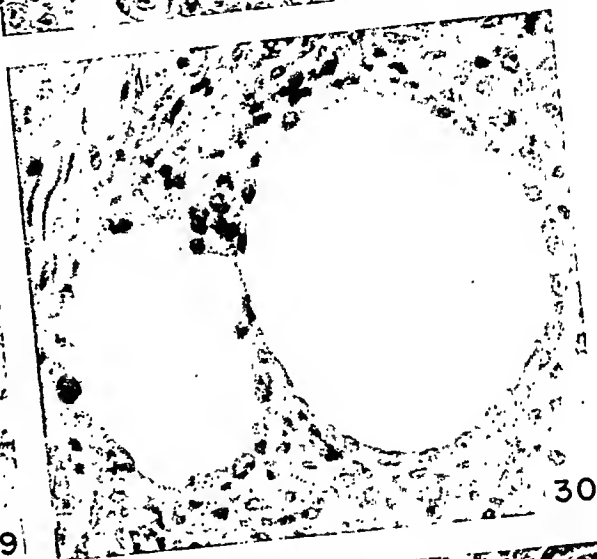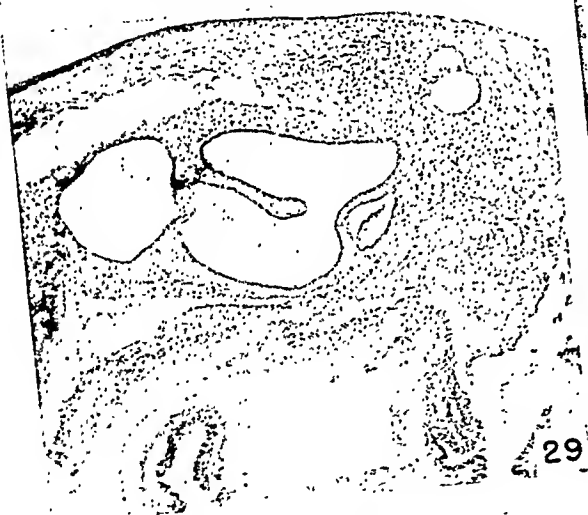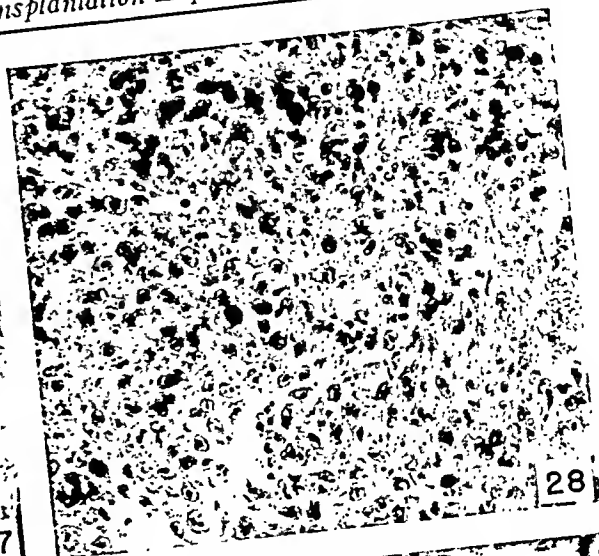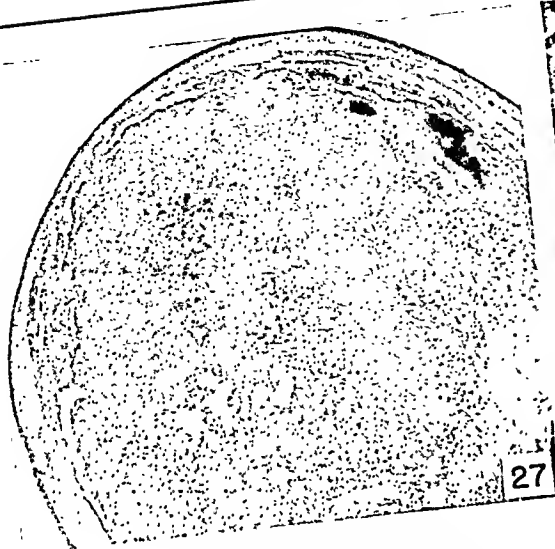
Carcinoma of the Vaginal Wall in the Rabbit*

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The characteristic distribution of uterine cancer in man with a high incidence in the cervix and a low frequency in the fundus does not obtain in the rabbit. Carcinoma of the uterine fundus is by far the most common of all neoplasms in the rabbit (1) whereas cervical cancer has not been observed in our laboratory nor has its occurrence been reported in the literature. The absence of a squamo-columnar junction in the rabbit's cervix suggests an anatomical basis for the species variation. Unlike the situation in man where columnar and squamous epithelium meet in the region of the external os, the columnar epithelium of the rabbit's fundus continues uninterruptedly over the cervix and down the vaginal wall to a junction with squamous epithelium at about the level of the urethral meatus. The object of the present paper is to report three cases of epidermoid cancer at this site. The occurrence of cancer at the squamocolumnar junction in both man and the rabbit despite its different location in the two species emphasizes the significance of the junction as a predisposing factor in carcinogenesis.

MATERIALS AND METHODS

The organization and management of the colony of rabbits in which the tumors occurred have been described in detail elsewhere (2). It should be noted, however, that the colony is maintained in active breeding service, the pedigrees and life histories of all animals are known and all abnormalities in behavior or general health are investigated. From September, 1931 to February, 1947,

the extent of the present report, the population was made up of 14 pure breeds, including the Belgian, Beveran, Chinchilla, Dutch, English, Havana, Himalayan, Polish, Rex, Sable and Silver Marten, Siamese Sable, French Silver and Tan breeds, and numerous hybrid lines.

The animals of this colony are subjected to weekly examination with particular attention directed toward a search for neoplastic foci. When tumors are found, biopsies are performed at frequent intervals and the natural history of the growth is followed to its termination. In none of the present cases, however, was the presence of the vaginal tumor discovered during life. In all three instances, the animals bore adenocarcinomas of the uterine fundus and had been under close observation supplemented by frequent laparotomy during the year preceding death. Despite such study, no clinical signs indicative of a co-existing vaginal tumor were observed.

Two of the tumors were transplanted after death, utilizing the anterior chamber of the eye as an inoculation site. The technic employed has been described (8).

INCIDENCE

During the 16 year period covered in the present report, approximately 1,100 female rabbits more than 2 years of age came to autopsy and, in each instance, the vagina and cervices were examined. The failure to find cervical growths acquires further significance in view of the fact that the rabbit's uterus is bicornate and contains 2 cervices. Thus, 2,200 cervices were examined without the discovery of a single tumor.

In contrast 3 tumors were found arising at the vaginal squamo-columnar junction. All were epidermoid carcinomas and, in each instance, metastasis had caused the death of the animal. It is of

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DESCRIPTION OF FIGURES 1 TO 4
All sections were stained with hematoxylin and eosin.

FIG. 1.—Section of fundic adenocarcinoma in rabbit X773-3. Mag. × 75.

FIG. 2.—Section of epidermoid carcinoma at the squamocolumnar junction in the vaginal wall of rabbit X773-3. Mag. × 35.

FIG. 3.—Section of metastatic epidermoid carcinoma in pleura of rabbit X773-3. Mag. × 35.

FIG. 4.—Section of metastatic epidermoid carcinoma in lung of rabbit X773-3. Mag. × 35.

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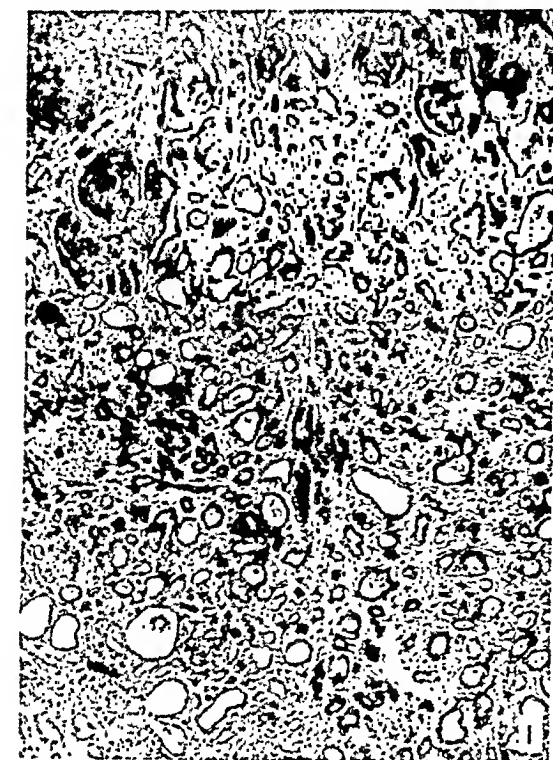
Figs. 1-4

interest that 3 extragenital epidermoid carcinomas were discovered in other female rabbits of this same series. One originated in the skin of the cheek (6), another in the skin of the tail and a third in a nipple. Thus, 3 out of 6 epidermoid carcinomas found in the rabbit arose at the vaginal squamo-columnar junction suggesting an increased susceptibility of this region over other bodily parts.

Age.—The ages of the 3 tumor-bearing animals at the time of death were 4 years, 11 months; 5 years, 2 months; and 5 years, 6 months. It should be emphasized that the given ages represent the time of death or of complete autonomous development of the tumors and that nothing is known with reference to the age at inception or the duration of the period of development. Postmortem examination was carried out on 29 females of the same age group during the period of study, indicating an incidence of the tumors in the group of approximately 13 per cent.

Breed.—One of the tumor-bearing animals was a purebred Havana: the others were complex hybrids. The ancestry of one hybrid involved an Havana male that had sired a parent of the purebred doe but the second hybrid bore no genetic relationship. The first hybrid contained Dutch and Polish blood as well, whereas the other was derived from a series of Belgian, Tan and Chinchilla crosses.

Constitution.—All 3 animals had been bred for constitutional study and were known to carry and to transmit genetic abnormalities. The Havana doe represented a concentration in heterozygous form of a variety of hereditary variations under study, the most important being arterio-sclerosis of Monckeberg type, renal aplasia, bile duct adenomas and a uterine anomaly expressed either as a unicornate organ or as an incomplete fusion of horns. The Polish hybrid transmitted cretinism (7) and dwarfism (3); the latter resulting in a diminution of the physique of the animal due to partial expression of the recessive character. The third rabbit belonged to a line known to carry factors concerned in the genesis of mammary cancer. In addition she had been found to transmit splenic reduplication (a condition characterized by the presence of 2 fully formed spleens), a pigmentary deficiency associated with maintenance of downy, infantile fur into adult life and a progressive disorder resulting in premature senility.

Despite such an array of constitutional variations, it should not be assumed that the tumor-bearing animals differed from other rabbits of the colony in this respect, or represented a more abnormal stock than the population of other colonies. In our experience, all rabbits subjected to inbreeding and sufficient study are found to be abnormal in some respect or to transmit some type of physical or functional variation.

The tumor-bearing animals did not share genetic variations of similar type and the role played by constitutional variation in the etiology of the tumors cannot be evaluated from present data.

Fundic adenocarcinoma.—Adenocarcinomas were present in the uterine fundi of all of the animals bearing vaginal tumors. It should be noted, however, that 23 or 79 per cent of the 29 animals in the same age group were similarly affected and the significance of the association may relate to age rather than to some other factor. Widespread endocrinological lesions comparable to those found in rabbits subjected to the long-continued administration of estrogenic substances are present in all animals bearing fundic cancers and it is assumed that the hormonal changes may be of importance in their genesis. Conceivably, the same factors may be operative in the etiology of the vaginal tumors.

Mammary cancer.—Two of the animals showed breast changes comparable with those found in the developmental stages of mammary cancer (2, 4). In one instance the disorder had progressed to the stage of multiple papillomas while in the other, the histological appearance of the tissue sectioned after death of the animal was indistinguishable from cancer. It seems probable that the breast changes in these cases relate to the same hormonal factor concerned in the genesis of the fundic tumors and that their association with vaginal cancers may be on the same basis.

Toxemia of pregnancy.—All 3 animals in this series had recovered from one or more attacks of toxemia of pregnancy. The nature of this disorder in the rabbit has been described (5) and its association with fundic adenocarcinomas noted (1).

DESCRIPTION OF FIGURES 5 TO 10

FIG. 5.—Section at edge of metastatic epidermoid carcinoma in liver of rabbit X773-3. Mag. × 35.

FIG. 6.—Section of fundic adenocarcinoma in rabbit X7634-3. Mag. × 75.

FIG. 7.—Section of vaginal wall at squamocolumnar junction of rabbit X7634-3. Mag. × 14.

FIG. 8.—Higher power view of epidermoid carcinoma shown in previous photograph. Mag. × 250.

FIG. 9.—Section of metastatic epidermoid carcinoma in diaphragm of rabbit X7634-3. Mag. × 250.

FIG. 10.—Section of metastatic epidermoid carcinoma in mediastinal lymph node of rabbit X7634-3. Mag. × 250.
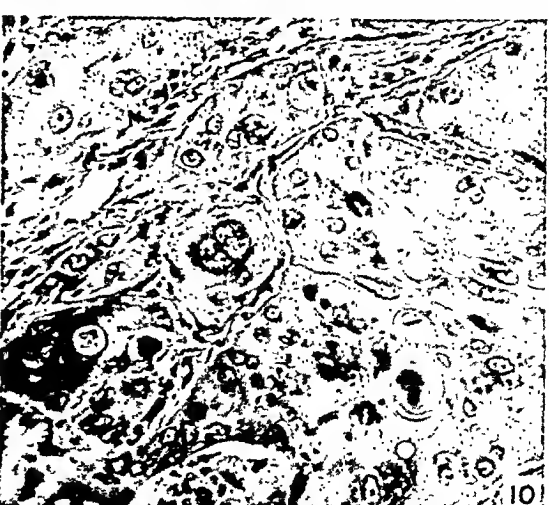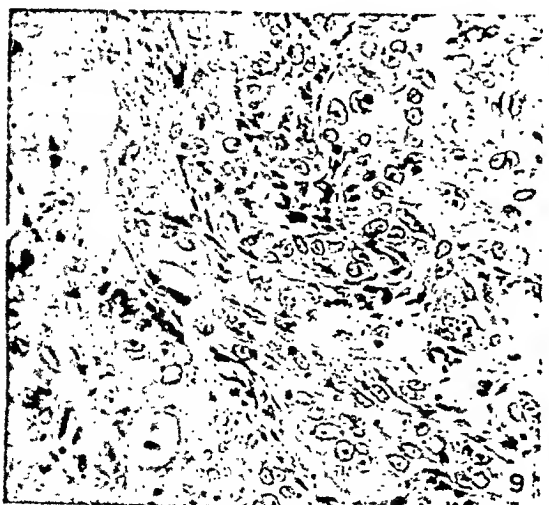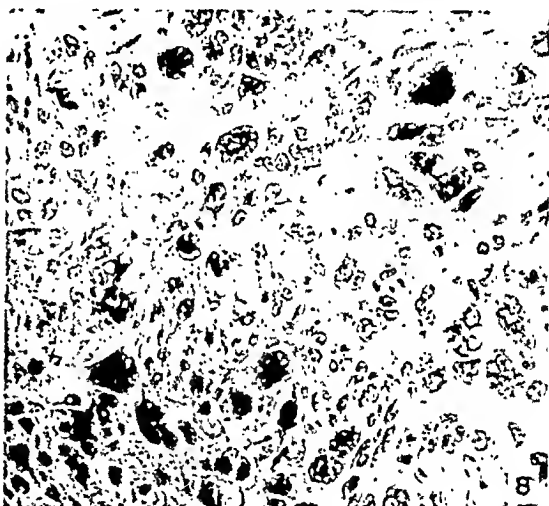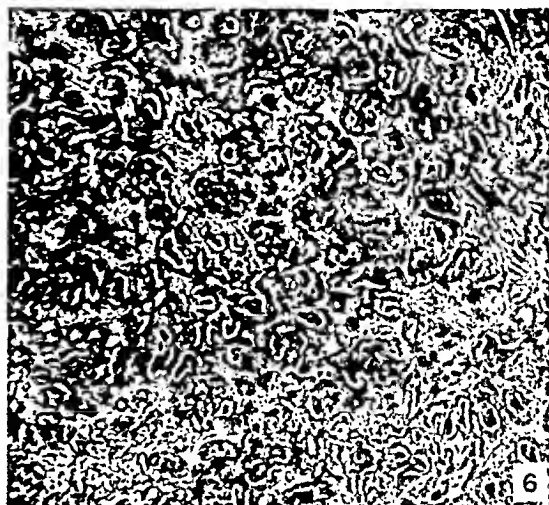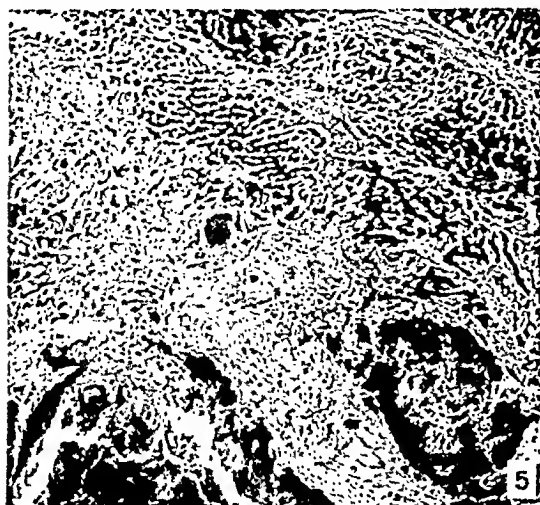
FIGS. 5-10

It would appear to occupy an analogous position in relation to vaginal tumors. The assumption has been made that the liver damage incident to toxemia of pregnancy results in destruction of the cells normally concerned in the inactivation of estrogenic hormone, and that, as a result, the hormone piles up in the circulation to a carcinogenic level.

CLINICAL HISTORY AND PATHOLOGY

The rabbit, X773-3, a Dutch-Polish hybrid, was first bred at the age of 6 months, and 13 out of the 23 matings carried out during the ensuing $3\frac{1}{2}$ year period proved fertile, resulting in a total of 46 living progeny. The gestation period following the 10th fertile mating was complicated by eclampsia and terminated in a dead-born litter, but no other irregularity distinguished the breeding history of the animal. Approximately a year later, a mass was detected in the uterine fundus and its identity as a developing adenocarcinoma confirmed by laparotomy and histological examination. The animal remained in good condition until a month before death and then, following a rapid weight loss, died at the age of 5 years, 2 months.

At autopsy, confluent masses of soft, necrotic tumor were found arising from the endometrium throughout both uterine horns, and in multiple areas, the growths had invaded and replaced the muscular wall. A firm mass of tumor tissue of different color and consistency was discovered in the posterior vagina opposite the urethral meatus and similar but smaller nodules were scattered irregularly throughout the upper vaginal wall. Metastatic tumor tissue with comparable gross characteristics was present in the omentum, liver, diaphragm, pleura, pericardium and almost completely replaced the lung.

The breeding history of the Belgian hybrid, X7634-3, also began at the age of 6 months and terminated with the discovery of a fundic tumor 3 years later. During this period 7 out of 20 matings were fertile and resulted in 32 living progeny. The course of 2 early pregnancies was associated with acetone breath, urine retention and fetal death but severe eclampsia with convulsions did not occur.

Three laparotomy examinations were made during the 2 year period from discovery of the uterine tumor to death. In each instance, tumor tissue was removed for microscopic study and transfer. Histologically, the tumor presented the usual picture of developing fundic adenocarcinoma. Autologous and homologous transfers were successful and the growth rates corresponded with the developmental stage of the tumor at the time of biopsy.

Several mammary biopsies were performed in this interval to investigate shotty nodules. Histological examination showed cystic disease and papillomas characteristic of the early stages of mammary cancer development in this line.

During the course of physical examination, about the middle of the fifth year of life, the animal's distended bladder was ruptured under pressure of palpation. She was killed immediately and a postmortem examination carried out. The uterus was distended by masses of confluent fungating tumor associated with widespread muscular invasion but without peritoneal extension. A mass of firm, almost cartilaginous tumor encircled the vagina at the squamo-columnar junction and extended upward to the cervix. Small nodules were present in the wall of the urethra and the bladder mucosa was diffusely thickened. In the region of rupture, a mass of tumor protruded into the bladder lumen and extended through the musculature into the peritoneum. Metastatic tumor was found in the inguinal, preaortic and mediastinal lymph nodes, in the diaphragm and in the lung. Other organs were not involved.

The Havana doe, HA550-2, was first bred at the age of 5 months and remained in breeding service for $2\frac{1}{4}$ years. Seven out of 19 matings proved fertile but 2 of these terminated in fetal resorption and a third in mild toxemia with fetal death. The 4 normal pregnancies resulted in 18 living young.

A laparotomy was performed, toward the middle of the third year of life, to investigate a mass noted in one uterine horn. This proved to be a retained placenta but a pronounced endometrial hyperplasia was found and it is of interest from the viewpoint of pathogenesis that a second laparotomy performed 3 months later disclosed a

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DESCRIPTION OF FIGURES 11 TO 16

Fig. 11.—Section of fundic adenocarcinoma in rabbit HA550-2. Mag. × 75.

Fig. 12.—Section of anaplastic epidermoid carcinoma at the squamocolumnar junction in the vaginal wall of rabbit HA550-2. Mag. × 250.

Fig. 13.—Section of aorta embedded in metastatic tumor from rabbit HA550-2. Mag. × 14.

Fig. 14.—Section of metastatic epidermoid carcinoma in inguinal lymph node of rabbit HA550-2. Mag. × 250.

Fig. 15.—Section of metastatic epidermoid carcinoma in lung of rabbit HA550-2. Mag. × 250.

Fig. 16.—Section of metastatic epidermoid carcinoma in femoral bone marrow of rabbit HA550-2. Mag. × 250.
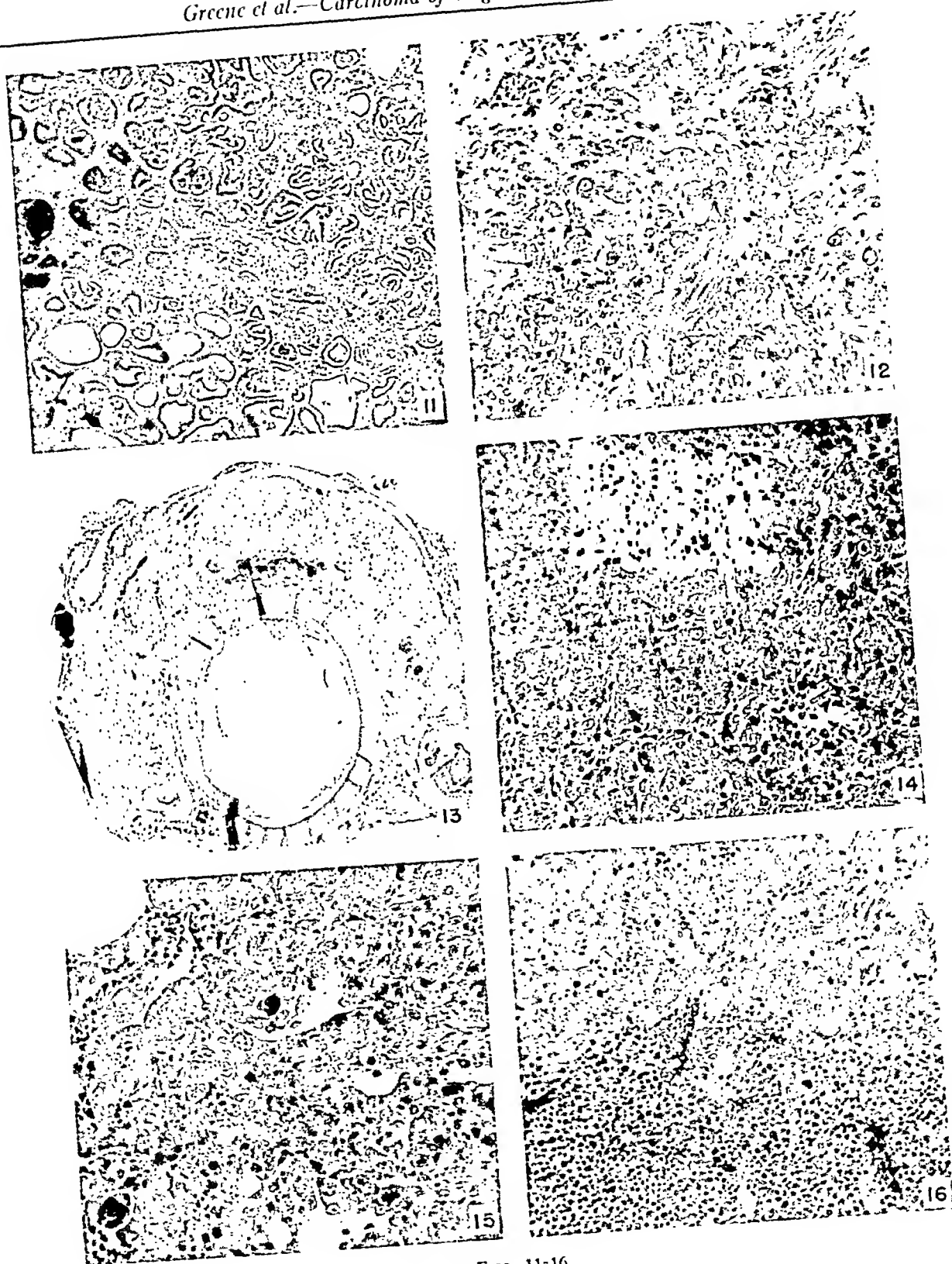
FIGS. 11-16

small endometrial tumor. The development of this tumor was followed throughout the remaining 2 years of life by means of serial biopsies with histological study and transfer of the tissue.

Mammary changes were noted soon after discovery of the uterine tumor. These were also followed by frequent biopsy and the sequence of changes progressing from simple cystic disease through papillomas to morphological cancer was observed. Despite the presence of all of the histological alterations generally considered characteristic of cancer, transfer of the breast tumor to normal animals was never successfully affected.

The rabbit died at the age of 4 years, 11 months, following a rapid weight loss over the period of several weeks. At autopsy, pelvic relations were identified with considerable difficulty. The uterus was replaced by a mass of soft necrotic tumor adherent to the abdominal wall and to the intestine. Innumerable firm nodules were scattered throughout the pelvic peritoneum and mesometrium and, on section, were discovered deep within the fundic tumor. Such nodules were comparable in all respects with the tumor mass found in the vagina. This tumor arose at the level of the urethral meatus, involved the upper two-thirds of the vagina, the urethra and bladder, and extended posteriorly to form a large mass in the pouch of Douglass. Extensions of this mass completely embedded the rectum and the abdominal aorta. The kidneys were hydronephrotic and contained large metastases. Metastases were also present in both ovaries, the spleen, liver, diaphragm, lung, mediastinal nodes, bone marrow and spinal muscles Secondary findings at autopsy were the mammary tumor previously mentioned and a large bile duct adenoma involving the greater part of the left half of the liver.

The histology of the tumors is illustrated in the accompanying figures (Figs. 1 to 17). The tumor found in the rabbit X773-3 was a well differentiated epidermoid carcinoma. The growth in X7634-3 was less differentiated and that in HA550-2 was the most anaplastic of the group. The metastases in all of the animals were epidermoid in type and were derived from the vaginal carcinoma. No metastases from the fundic or mammary tumors were found.

TRANSPLANTATION OF THE VAGINAL TUMORS

Tumor tissue from the animals X7634-3 and HA550-2 was successfully transplanted to the anterior chamber of the eye in normal rabbits. Transfer of the X773-3 tumor was not attempted.

The tissue used for the transfer of the X7634-3 tumor was derived from a mediastinal lymph node. The percentage of takes increased from 50 per cent in the first generation to 80 per cent in the seventh where passage of the tumor was discontinued. The growth rate varied greatly and was not consistently increased with continued transfer. In some instances, the tumor completely filled the anterior chamber of the eye in 27 days while in others the growth had not reached a quarter of this size by the 150th day. Vascularization occurred early in both types of growth, and apparently played no part in the observed variation. Invasion of the iris was also an early occurrence but extension into the vitreous humor did not occur. The histology of the transplants is shown in Figs. 19 and 20.

Transplantation of the mammary tumor from this animal was unsuccessful. Transfer of the fundic cancer resulted in 100 per cent of takes but the growth rate was extremely slow, the tumor consistently filling one half of the chamber in 230 days.

A metastatic liver nodule was utilized in the transfer of the HA550-2 tumor. A hundred per cent of takes was obtained in the first generation and the incidence ranged from 80 to 100 per cent throughout the 14 serial passages of the tumor. The growth rate was rapid and, as a rule, the anterior chamber of the eye was filled by the 20th day. A feature of interest in the transfer of this tumor was the fact that, in many cases, growth occurred without vascularization and continued until the chamber was filled without the appearance of an independent blood supply. Apparently the cells multiplied and grew in the manner of a tissue culture, deriving their nutrient directly from the aqueous humor. Similar observations have been made in the case of other anaplastic cancers and it seems possible that the disorganized appearance of such tumors may be due to a lack of an ability of their cells to stimulate the adequate growth of stroma. The histology of the transplants of this tumor is shown in Fig. 18.

DESCRIPTION OF FIGURES 17 TO 20

FIG. 17.—Section of metastatic epidermoid carcinoma in lung of rabbit HA550-2. Mag. × 250.

FIG. 18.—Section of anterior chamber transplant of epidermoid carcinoma from rabbit HA550-2. Mag. × 250.

FIG. 19 and 20.—Sections of anterior chamber transplants of epidermoid carcinoma from rabbit X7634-3. The bizarre cells seen in Fig. 20 characterized older transplants. Mag. × 250.
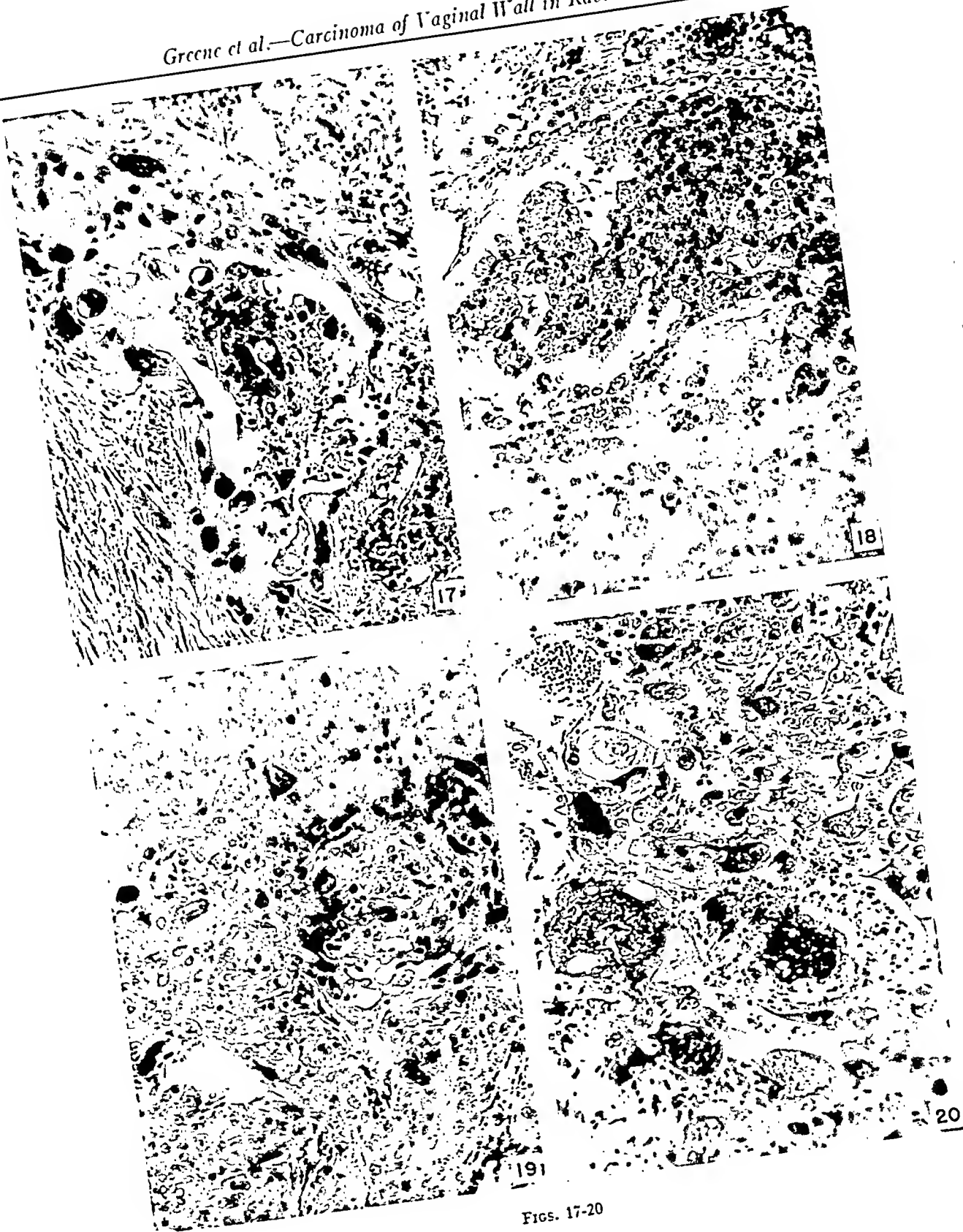
FIGS. 17-20

Transfer of the breast tumor of this animal with material obtained at autopsy was unsuccessful. Transfer of the fundic uterine tumor with material taken at autopsy gave rise to 40 per cent of takes in 3 transplanted generations and the growth rate was slow, a period of 160 days being required for the filling of the chamber. The behavior of the fundic tumor at this transfer was in marked contrast to the behavior of tissue obtained from it at a biopsy 4 months before death. At that time, 92 per cent of takes were obtained in 11 transplanted generations and the anterior chambers were consistently filled with tumor by the 45th day. The possible influence of the developing vaginal tumor on the biological behavior of the fundic growth will be considered in detail in a subsequent paper reporting transplantation experiments with a series of multiple tumors in the rabbit.

DISCUSSION

There is little question of the analogy between the squamo-columnar junction on the human cervix and the epithelial junction in the vaginal wall of the rabbit. It is true that both the columnar epithelium of the endocervix and the squamous epithelium of the vagina in man are coelomic in origin, while in all probability, the squamous epithelium of the vaginal wall of the rabbit represents the lining of the urogenital sinus and being derived from ectoderm is embryologically analogous to the external skin. Thus, in man the junction is one between epithelia of common embryological derivation while, in the rabbit, it is actually a meeting of mesothelium and ectodermal epithelium. This difference cannot be considered of great significance in the present connection. The junction in the two species represents the joining of epithelia of different morphological type. Both are subjected to the irritative action of vaginal secretions and the trauma of parturition; neither is stable but fluctuates in location in response to inflammatory stimuli and in effect constitutes a zone of epithelial restlessness.

The occurrence of carcinoma at the squamocolumnar junction in the rabbit despite its different location in this species emphasizes the significance of the junction in the incidence of cervical carcinoma in women.

SUMMARY

Three epidermoid carcinomas arising at the squamocolumnar junction of the vaginal wall of the rabbit have been described. The relatively high frequency of cancer at the squamocolumnar junction in both man and the rabbit despite its different location in the two species emphasizes the significance of the junction as a predisposing factor in carcinogenesis.

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Strain Differences in Response to Diethylstilbestrol and the Induction of Mammary Gland and Bladder Cancer in the Rat

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For the past 20 years, the field of experimental cancer research has been largely dominated by studies on the occurrence, production, or prevention of mammary cancer in the mouse. The development through inbreeding and selection of numerous strains of mice in which these neoplasms occur spontaneously with a relative high frequency and the economy of feeding and housing mice in preference to other laboratory animals have been factors contributing to this situation. So predominate is this particular form of malignant disease that mice that fail to develop it have become known as non-tumor mice, irrespective of any further tumor history or the ease with which they respond to various tumor-inducing agents. The controversy over the hereditary nature of these tumors was largely explained by the discovery of Little and his co-workers (8) and of Korteweg (12) of an important extrachromosomal or milk influence. This was demonstrated by significant differences in the incidence of mammary tumors in the F_1 progeny of reciprocal crosses between high and low mammary cancer stocks. Bittner (2, 3) demonstrated further, that foster nursing of the progeny of cancerous mothers by low mammary cancer stock mothers largely prevented the occurrence of mammary cancer in these foster-nursed females. More recently Bittner (4) has shown that feeding the filtrate from macerated lactating mammary glands obtained from mice of high mammary cancer stock caused the foster-nursed daughters to develop mammary cancer 8 to 10 months later. Green, Moosey and Bittner (9) have further demonstrated the production of antibodies and antiserums, which are capable of neutralizing or inactivating the centrifugates of mouse mammary cancer, while antiserums produced similarly in response to normal mouse tissue are ineffective. Thus an important factor in mouse mammary cancer development appears to be a self-perpetuating, exogenous body analogous to a virus.

Early experiments in castration demonstrated the relationship between the ovarian hormones and the development of mammary cancer in the mouse. Prepubertal ovariectomy inhibited the development of mammary tissue and prevented the occurrence of mammary cancer while the injection of estrogens caused the mammary cancers to appear earlier than they did in untreated females. Lacassagne (10, 11) showed that the injection of estrogens in male mice from birth or an early age caused them to develop mammary cancer in the same frequency as their sisters. These findings have been confirmed by many investigators as indicated in a recent survey of the literature on the subject by Gardner (7). It seems however, that the female sex hormones alone are insufficient to cause the development of mammary cancer in some mice that lack the hereditary factors. Thus the development of mammary cancer in the mouse appears to be dependent upon three separate etiological factors; namely, hereditary, extrachromosomal or milk, and hormonal. This anomalous situation has given impetus to a search for similar factors in the etiology of other types of neoplasia in the mouse and of mammary cancer in other species, including man. So far, the search has failed to identify in other species a factor analogous to the milk influence in mammary cancer of mice.

The rat has been considered peculiarly insusceptible to mammary cancer but, although Curtis, Bullock and Dunning (5) reported only 2 mammary cancers in a colony of nearly 9,000 female rats of tumor age, the animal has not proved resistant to the induction of mammary cancer by excessive estrogenic stimulation. The contrast between the relatively low frequencies of induced mammary cancer reported by McEuen (13) and Eisen (6) and the high frequency obtained by Geschickter and Byrnes (8) and Nelson (14) would suggest the operation of

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possible hereditary factors, since these investigators employed different strains of rats. McEuen reported only 2 mammary cancers in 12 rats that survived for 500 days with daily injections of 30 γ of estrone and Eisen obtained 2 mammary cancers in 103 rats that survived for 242 days with implantations of 1 to 20 mgm. of crystalline estradiol dipropionate in paraffin. In contrast, Nelson observed 63 mammary cancers in 103 treated rats that survived 300 days and longer. Geschickter and Byrnes reported 202 rats with mammary cancer from 555 rats treated with various doses of estrone and stilbestrol and prescribed that, to produce mammary cancer in the rat, the dose of estrogen must be well beyond the physiologic limit (10 or more times the threshold dose) and the treatment continuously applied for a period of months (30 or more times the duration of normal estrus). Since the dose of estrogen employed by McEuen and by Eisen met these requirements, there would seem to be some fundamental difference in the rats used for these investigations. Obviously, unphysiological estrogenic stimulation is a factor in the etiology of mammary cancer in the rat as well as in the mouse. Here would seem to be an excellent opportunity to determine further, whether or not hereditary or milk factors predispose rats to estrogen-induced mammary cancer.

It is the purpose of the present paper to report the response of 3 inbred lines of rats to unphysiological stimulation from diethylstilbestrol. A later communication will deal with the response of reciprocal F_1 hybrids between 2 of these lines to an effective mammary cancer-inducing dose of diethylstilbestrol.

MATERIALS AND METHODS

Pedigreed rats of 3 distinct lines, Fischer line 344, Copenhagen line 2331, and A $\times$ C line 9935, were used for this investigation. They received the laboratory stock diet (Friskie Dog Pellets) supplemented with a green vegetable once a week. The cancer history of the ancestors of these three lines is known for 40, 31 and 27 brother-by-sister generations, respectively, and no spontaneous mammary cancers were observed. Each series consisted of 30 males and 30 females from each line, which at the start of the experiment were between 3 and 4 months of age.

In Series I, pellets weighing between 15 and 25 mgm. of compressed crystalline diethylstilbestrol[1]

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[1] Supplied through the courtesy of Dr. D. F. Robertson of Merck and Co., Rahway, N. J.

were implanted in the scapular region by means of nasal forceps inserted through an incision in the skin of the lower back. The incision was closed with a wire staple. The rats were weighed and inspected for mammary tumors every 2 weeks. Any rat that gained appreciably for 2 successive weighings, or any male in which the testicles descended, or any female found to be in a diestrous phase, was reimplanted with another pellet of diethylstilbestrol. At death any remaining pellet was removed, weighed and subtracted from the weight of diethylstilbestrol that had been administered to the rat. At the postmortem examination, a thorough inspection for gross tumors was made and gross sections of mammary gland, thymus, liver, kidney, adrenals, urinary bladder, sex glands, and pituitary were preserved for microscopic examination.

In Series II, a similar group of rats were implanted in the scapular region with pellets composed of 75 per cent cholesterol and from 4 to 15 mgm. of diethylstilbestrol. The diethylstilbestrol in this series was much more slowly absorbed and no reimplantations were necessary to keep the rat in a constant state of hyperestrinism for the remainder of its life. It was, however, impossible to determine the amount of diethylstilbestrol actually absorbed from the pellets because at the death of the animal, after many months in the rat, some of the pellets weighed the same or more than when they were implanted. Otherwise, these rats were treated similarly to those of Series I.

RESULTS

The results are summarized briefly in Tables I and II and shown graphically in Figs. 1 and 2. The rats of Series I represented in Table I and Fig. 1 showed considerable variation in survival and in the absorption of diethylstilbestrol. The 17 Fischer line 344 females on which complete records were obtained survived for an average of only 67 days and had absorbed 19 mgm. of crystalline diethylstilbestrol or an average of 0.26 mgm. per rat per day. The average body weight fell from 120 gm. to 110 gm. in the first 2 weeks and remained low. One female survived for 143 days, having absorbed 34 mgm. of diethylstilbestrol. All of these females died with pyometra and in most cases peritonitis was evident. The Fischer line 344 males lived twice as long, or an average of 136 days, absorbing an average of 29 mgm. of diethylstilbestrol at the rate of 0.2 mgm. per rat per day. Their average body weight fell from 167 to 144 gm. within 2 weeks and only 4 of the rats survived for 6 months. The Copenhagen line 2331 and A $\times$ C line 9935 rats absorbed the diethyl-
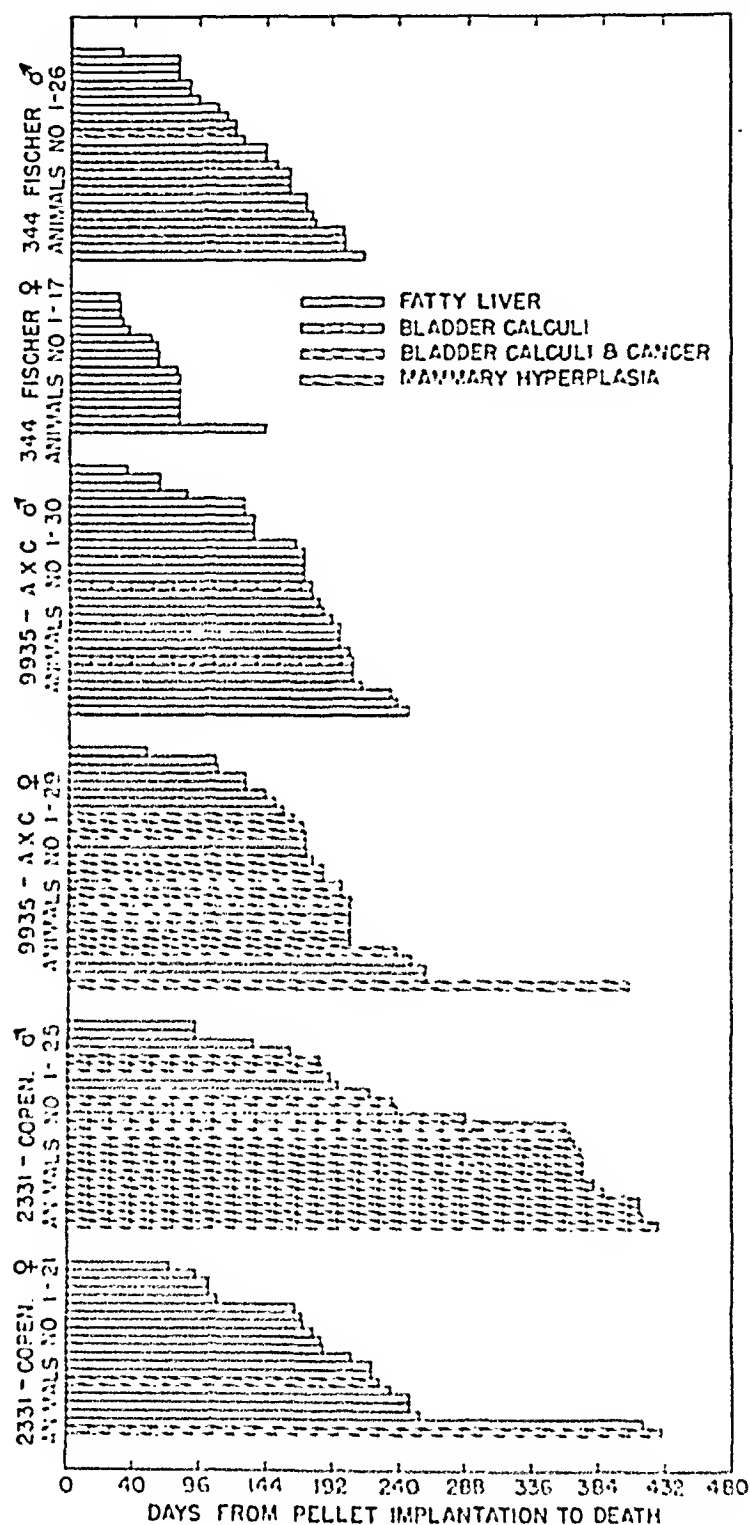
FIG. 1. Survival period and tumor history of rats of each strain with pellets of crystalline diethylstilbestrol implanted in subcutaneous tissues of scapular region. (Each rat is represented by a bar, length of which shows the period of survival in days, with postmortem findings indicated by proper shading.)
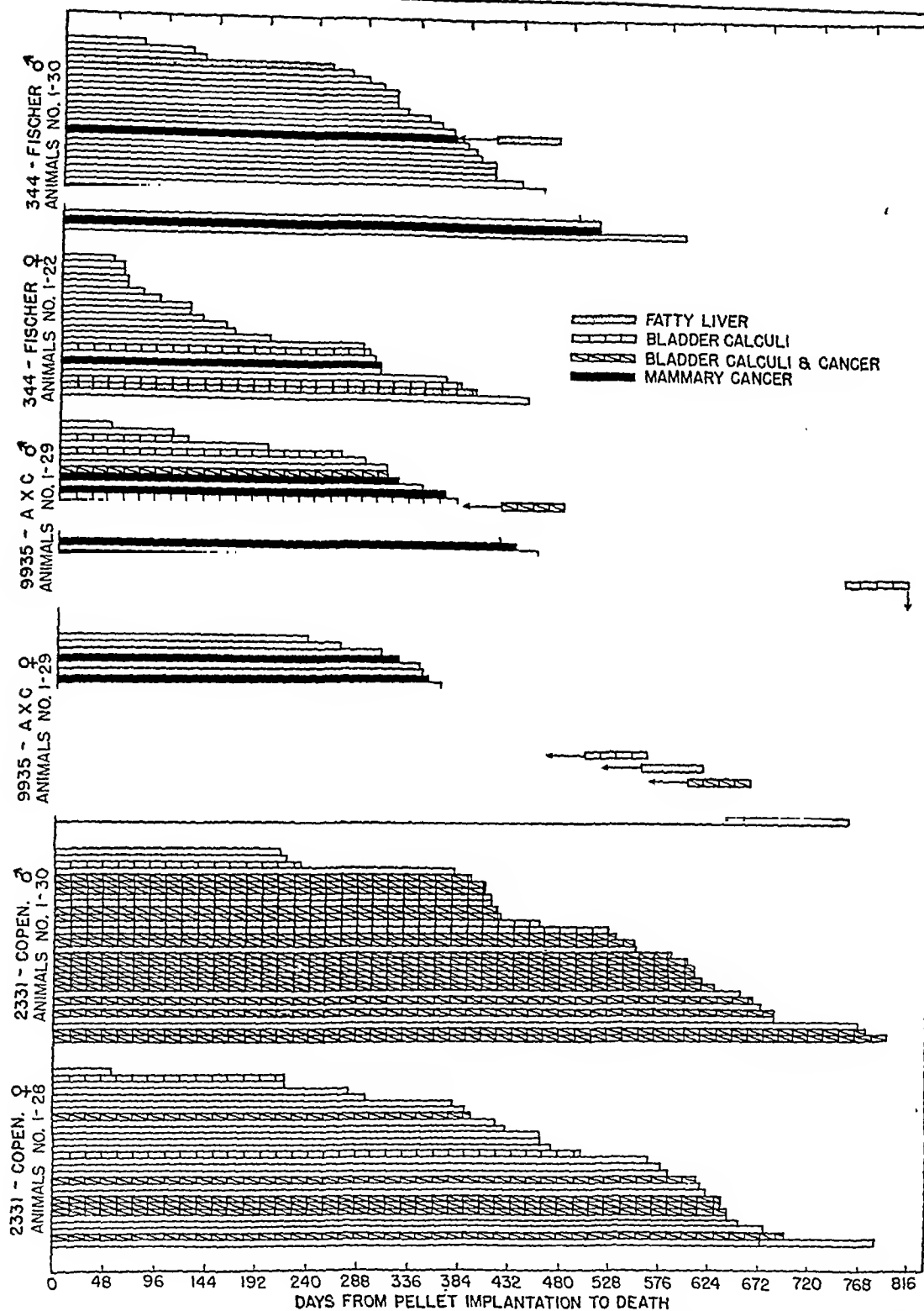
FIG. 2.—Survival period and tumor history of rats of each strain with cholesterol pellets containing 25 per cent diethylstilbestrol implanted in subcutaneous tissues of the scapular region. (Each rat is represented by a bar, length of which shows the period of survival in days, with post-mortem findings indicated by proper shading. In the cases where rat had mammary cancer and another of the pathological conditions, the latter is indicated by a box with proper shading connected by an arrow.)

stilbestrol more slowly and survived the treatment longer. There was less difference between the A × C males and females, but the female had a longer average survival because one female lived for 406 days, as shown in Fig. 1. The average survival period was 167 days for the males and 186 days for the females, while the former absorbed 24 mgm. of diethylstilbestrol and the latter an average of 25 mgm. The average body weight dropped from 142 gm. to 117 gm. in the males and in the females from 116 gm. to 101 gm. within 2 weeks after the pellets were implanted. The Copenhagen line 2331 males survived the treatment longer than any of the other rats— an average of 289 days compared with 200 days for the females. The former absorbed 38 mgm. of diethylstilbestrol and the latter 31 mgm. The average body weight of the males fell from 122 gm. to 94 gm. in the first 2 weeks and that of the females from 115 gm. to

in Fig. 3. The albino rat on the right, a Fischer line 344 male, had absorbed 33 mgm. of diethylstilbestrol in 170 days. The pituitary in this rat weighed 400 mgm. The rat on the left was a Copenhagen female which had absorbed 34 mgm. of diethylstilbestrol in 183 days. The pituitary weighed 60 mgm. The photograph also shows the pellets *in situ*.

One of these large hemorrhagic pituitary adenomas from a Fischer line 344 male was transplanted into the subcutaneous tissues of 6 males of the same line in which diethylstilbestrol pellets had been previously implanted. All of the grafts grew progressively until the hosts died from the effects of the rapid absorption of diethylstilbestrol with large pituitaries *in situ*. Some of the subcutaneous growths attained a diameter of 1.5 cm. and weighed nearly 1 gm. A section through one of these subcutaneous growths is shown in Fig. 9. It has the

TABLE 1: NUMBER OF RATS OF EACH STRAIN, AVERAGE DAY OF DIETHYLSTILBESTROL, AVERAGE SURVIVAL AND PITUITARY WEIGHT, AND NUMBER OF RATS WITH OTHER PATHOLOGICAL LESIONS

No. of rats	Sex	Strain	Av. no. days until death	Av. amt. of stilbestrol (mgm.)	Av. daily absorption of stilbestrol (mgm.)	Average pituitary wt. (mgm.)	Fatty liver	Mammary hyperplasia (hyperplasia/neoplasia)	Bladder calculi
26	♂	Fischer	156	25.6	0.20	405	16	1	0
17	♀	Fischer	67	18.8	0.26	309	4	0	0
30	♂	A × C	167	24.1	0.14	165	0	0	2
29	♀	A × C	186	25.4	0.14	161	0	13	0
25	♂	Copenhagen	289	38.3	0.13	98	0	0	16
21	♀	Copenhagen	200	30.5	0.15	63	0	2	1

94 gm. Eleven males and 2 females survived for more than a year.

No mammary cancers were observed in these rats, although considerable hyperplasia of the mammary tissue was noted in 13 A × C females, also in one Fischer male and 2 Copenhagen females. The most conspicuous differences were observed in lesions of the pituitary, liver and bladder. The Fischer line 344 males and females without exception had large hemorrhagic pituitary adenomas, which in some cases weighed as much as 500 mgm. The Copenhagen line 2331 rats had enlarged pituitaries, which rarely became hemorrhagic and averaged less than 100 mgm., although they lived significantly longer than the Fischers and absorbed more diethylstilbestrol. The pituitaries in the A × C line 9935 rats were intermediate, rarely obtaining the size of those in the Fischers and usually exceeding those of the Copenhagens. A previous publication (16) noted a similar difference in response of the pituitary and also a distinct difference in the adrenals of A × C and Fischer castrated males to daily injections of 1 mgm. of diethylstilbestrol. The characteristic difference between the pituitary adenomas in the Fischer line 344 and the Copenhagen line 2331 rat is shown

characteristic large sinuses filled with red blood cells, is quite cellular with many mitoses and in many respects resembles a neoplasm, but it failed to grow upon transplantation into untreated rats of the same inbred line.

Extensive fatty infiltration of the liver was observed in 16 of the Fischer line 344 males and in 4 of the females. Fatty livers were not found in treated rats of the other 2 lines nor in untreated Fischer rats. A section through one of these livers is shown in Fig. 7.

The most striking lesion was observed in the bladder of the Copenhagen line 2331 male rats which had been treated for 6 months and more. The bladder was distended up to 10 times its normal size, and in many instances was packed with faceted calculi. These, usually smooth and rounded, but sometimes rough and crystalline, proved to be chiefly magnesium ammonium phosphate like those reported by Benjamin, Wilson and Leahy (1) for α-estradiol-treated rats. The walls of the bladder were usually thickened and papillary and in 14 of the 16 males with calculi, contained one or multiple papillomas. One female Copenhagen had bladder calculi and papilloma and two A × C males also had bladder calculi.
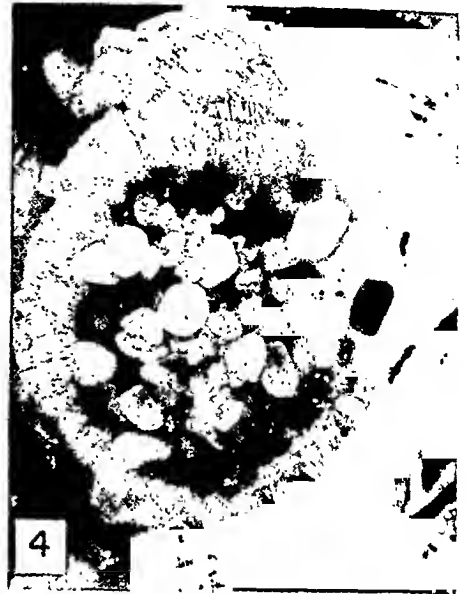
FIG. 3.—Pituitary adenoma and diethylstilbestrol pellets *in situ*. *White rat* = Fischer line 344 male which absorbed 33 mgm. of crystalline diethylstilbestrol in 170 days. *Agouti rat* = Copenhagen line 2331 female which absorbed 34 mgm. of crystalline diethylstilbestrol in 183 days.

FIG. 4.—Calculi exposed in the bladder of an A✕ C line 9935 rat 308 days after receiving a cholesterol pellet containing 12 mgm. of diethylstilbestrol.

FIG. 5.—A ✕ C line 9935 rats with induced mammary cancers. At right, female after 371 days with 5 mgm. of diethylstilbestrol in cholesterol pellet. At left, female after 377 days with 7.5 mgm. of diethylstilbestrol in cholesterol pellet.

FIG. 6.—A ✕ C line 9935 female with induced lymphosarcoma 322 days after receiving 4 mgm. of diethylstilbestrol in cholesterol pellet.
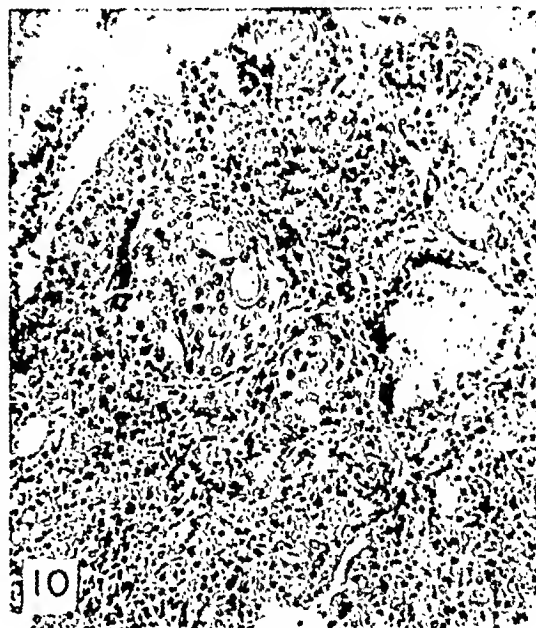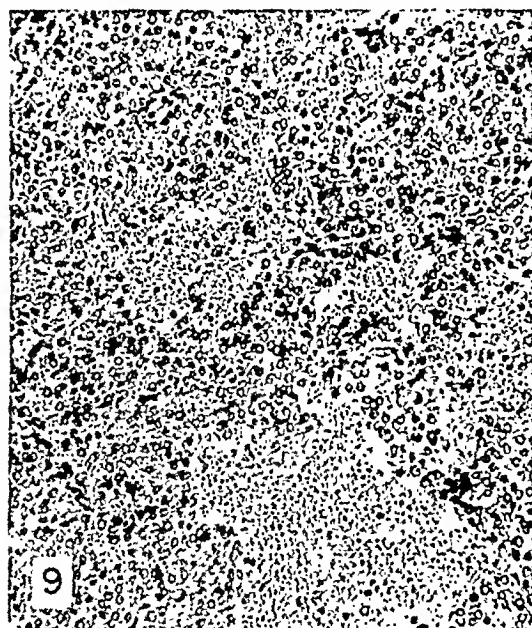
FIG. 7.—Section of a fatty liver from a Fischer line 344 rat which absorbed 33 mgm. of diethylstilbestrol in 177 days. Mag. × 125.

FIG. 8.—Section of a bladder papilloma in a A × C rat 308 days after it received 12 mgm. of diethylstilbestrol in a cholesterol pellet. Mag. × 50.

FIG. 9.—Section of a subcutaneous transplant of a pituitary adenoma from a Fischer line 344 rat, 198 days after transplantation. Mag. × 125.

FIG. 10.—Precancerous lesion from the mammary gland of an A × C male rat, 308 days after it received 14.0 mgm. of diethylstilbestrol in a cholesterol pellet. Mag. × 150.

Fig. 4 shows one of these bladders with the calculi exposed and Fig. 8 shows a section through one of the papillomas. Unlike the urinary calculi reported by Wilson, Benjamin and Leahy (19) in rats, and Schenken, Burns and McCord (15) in α-estradiol-treated C3H mice, these did not appear to be associated with inflammatory changes in the urinary tract. No calculi were found in the kidneys

or ureters and pathological changes were no more frequent in the kidneys of these rats than in those of the treated rats of the 2 other lines.

The rats of Series II, which received the pellets composed of 25 per cent diethylstilbestrol and 75 per cent cholesterol tolerated the treatment much longer as shown in Table II and Fig. 2. Half of the Fischer line 344 males and 4 of the females survived for a year. The majority of the rats of the two other lines lived more than a year and 2 A × C and 4 Copenhagen rats survived for more than 2 years. The Fischer and A × C females lost an average of only 2 to 4 gm. during the first 2 weeks after the pellets were implanted whereas the Copenhagen females lost 10 gm. The males lost from 16 to 20 gm. during the interval, but their weight gradually increased after the first 4 months. Very few fatty livers were observed, 2 in Fischer line 344 males, 1 in a Fischer and 1 in

A × C rats. The difference between the Fischer line 344 and A × C line 9935 rats is partly attributable, as shown in Fig. 2, to the differences in survival, but not entirely. The tumors observed in the Fischer line 344 rats were all solitary as shown in Table II, but in the A × C rats most of the tumors were multiple. Typical examples are shown in Fig. 5. The rat at the left of the photograph had 6 probably independent tumors and the one on the right had 3. Fourteen independent tumors were found in one female; and many of the rats had additional areas of extreme hyperplasia with ductal infoldings and squamous metaplasia as shown in Fig. 10, which were considered precancerous lesions. In the 22 A × C line 9935 female tumor-bearers, 89 tumors were observed or an average of 4 to the rat. In the 17 A × C males 39 tumors were described or an average of 2 to the rat. One A × C male had 5 gross tumors and one

TABLE II: NUMBER OF RATS OF EACH STRAIN, AVERAGE DOSE OF DIETHYLSTILBESTROL IN CHOLESTEROL, AVERAGE SURVIVAL, AND NUMBER OF RATS WITH MAMMARY GLAND CANCER AND BLADDER CALCULI AND CANCER

No. of rats	Sex	Strain	Av. no. days until death	Av. amt. of stilbestrol (*mgm.*)	Average pituitary wt. (*mgm.*)	Mammary gland		Bladder	
						No. of rats	No. of cancers	No. with calculi	No. with cancer
30	♂	Fischer	367	7.7	147	5	5	0	0
22	♀	Fischer	200	7.5	104	1	1	3	0
29	♂	A × C	388	7.4	155	17	39	6	2
29	♀	A × C	442	5.4	116	22	89	1	1
30	♂	Copenhagen	528	8.6	64	0	0	22	16
28	♀	Copenhagen	499	9.0	92	0	0	8	6

an A × C female. The pituitaries were enlarged but not as consistently or as rapidly, or to as great a magnitude as in the rats of the other series.

Bladder calculi were observed in 22 or 73 per cent of the 30 Copenhagen line 2331 males and in 8 or 30 per cent of the 28 females of this line. The first were observed in a male that died 231 days after the pellet was implanted. In Series I, the calculi were found consistently in the Copenhagen line 2331 males after 180 days. Six A × C line 9935 males and 1 female also had calculi and a few small crystals were observed in the bladder of 3 of the Fischer line 344 females. Bladder papillomas and grade I squamous cell cancer were observed in 16 of the Copenhagen line 2331 males and 6 of the females which had calculi. Two A × C line 9935 males and 1 female also had bladder papillomas and cancer.

In Series II, mammary cancer occurred in 22 or 85 per cent of the females and in 17 or 80 per cent of the males of A × C line 9935. One Fischer line 344 female and 5 Fischer males also had mammary cancers. No mammary cancers were found in the treated Copenhagen males or females, although they outlived the Fischer line 344 rats by several months and lived fully as long as the

other which was identified by microscopic examination of the mammary tissue. Fig. 6 shows an A × C female rat with a lymphosarcoma which arose in the superficial lymph nodes of the left groin breast and metastasized to the other superficial lymph nodes, mediastinum and lungs. Fig. 14 shows a section through this tumor. The mammary cancers[2] in the A × C rats included 107 papillary cystic adenocarcinomas (Fig. 11), 11 adenocarcinomas and squamous cell cancers or adenocanthomas (Fig. 13), 3 solid carcinomas (Fig. 12), 2 solid carcinomas with papillary areas and 4 that were unclassified. The mammary tumors in the Fischer line 344 rats included 1 solid carcinoma, 1 interductal carcinoma and 4 papillary cystic adenocarcinomas.

No pellet was found in the A × C female shown in Fig. 2, which outlived the others and died without a mammary cancer. Scantily developed mammary tissue and a small pituitary (10 mgm.) suggested that the pellet had ulcerated out a considerable time before. Postmortem examination revealed an extensive osteosarcoma of the uterus
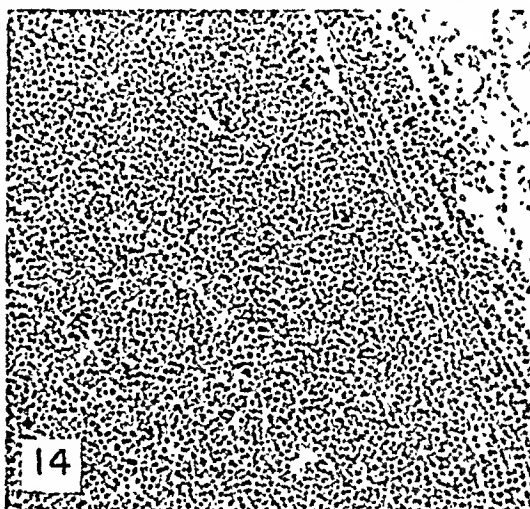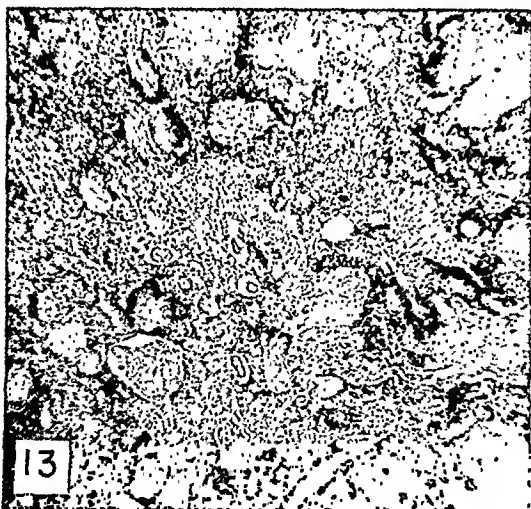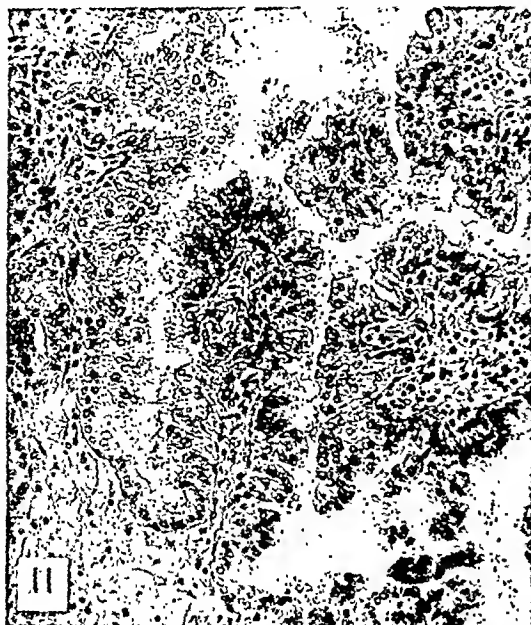
Fig. 11.—Papillary cyst adenocarcinoma from an A × C female rat, 348 days after it received 4 mgm. of diethylstilbestrol in a cholesterol pellet. Mag. × 150.

Fig. 12.—Mammary carcinoma from a Fischer line 344 female rat, 302 days after it received 7 mgm. of diethylstilbestrol in a cholesterol pellet. Mag. × 125.

Fig. 13.—Adenoacanthoma from an A × C female rat, 349 days after it received 4 mgm. of diethylstilbestrol in a cholesterol pellet. Mag. × 50.

Fig. 14.—Lymphosarcoma from an A × C female rat, 322 days after it received 4 mgm. of diethylstilbestrol in a cholesterol pellet. Mag. × 125.

with metastases to the lung and lymph nodes, which may have been related to the previous estrogenic stimulation. Another A × C female had a mixed tumor (chondrosarcoma and adenocarcinoma) of the ovary and a third, a carcinoma of the adrenal cortex. The latter probably resulted from the treatment, as it is a common sequel to estrogenic treatment in another strain of rats. Two A × C females had benign tumors of

the thymus and 1 a lymphosarcoma of the ileocolic mesentery, which were probably unrelated to the treatment, since they occur in untreated rats of this strain.

Two Fischer line 344 males had adenoma of the adrenal cortex, 1 an adenocarcinoma, and another had a hepatoma. A lymphosarcoma of the ileocolic mesentery was observed in 1 Fischer male, and a Fischer line 344 female had a squamous carcinoma

of the left ear, which were probably unrelated to the treatment.

Aside from the bladder carcinomas previously mentioned in the Copenhagen line 2331 rats, 1 female had a myosarcoma of the uterus; 1 male, a benign tumor of the thymus; 1 male, a lymphosarcoma of the ileocolic mesentery; and 1 male, a lymphocytic leukemia. The 3 last-mentioned neoplasms were probably unrelated to the treatment, although leukemia and lymphosarcoma are rare in untreated rats of this line.

DISCUSSION

Comparison of the results of Series I and II show that a slow continuous absorption of diethylstilbestrol is an effective mammary cancer incitant in some strains of rats, whereas a cyclical or more rapid absorption of even larger doses with a short intervening rest period is ineffective in rats of the same inbred line. Only 5 A × C line 9935 females and 3 A × C line 9935 males of Series I survived as long as the latent period previous to the observation of the first gross tumors in A × C rats of Series II, but no tumors could be identified by microscopic examination of their mammary tissue. These rats had absorbed from 20 to 39.1 mgm. of diethylstilbestrol. In a subsequent series, 4 A × C line 9935 rats survived for a year or more after the implantation of a single 20 to 25 mgm. pellet of crystalline diethylstilbestrol, plus 2 per cent methyl cellulose used as a binder without developing any mammary tumors.

The difference in response between the rats of the Copenhagen line 2331 and those of the other 2 lines that were tested, may be related to the manner of excreting the diethylstilbestrol and the formation of urinary calculi, or to the pre-existing disturbance in metabolism. However, the physiological processes resulting in formation of bladder calculi and bladder neoplasms in one line and mammary neoplasms in the other two are not mutually exclusive, as shown in Fig. 2, by 4 A × C rats with mammary cancer and bladder calculi. The mammary tissue of the Copenhagen rats of both series was less well developed than that of the rats of the two other lines, but areas of hyperplasia with lactating dilated ducts were commonly found and a few lesions which could be considered as precancerous were also observed. The mammary tissue of the Fischer line 344 rats seemed to be even more copiously developed than that in the A × C rats which responded with the higher percentage of mammary cancers. Possibly another estrogen or dose might reverse the reactions of these rats. For α-estradiol (17), the Fischer line

344 rats showed the highest threshold for vaginal estrus and an impaired ability for hepatic estrogenic inactivation, while the Copenhagen line 2331 rats exhibited a high threshold for vaginal estrus and the greatest ability for hepatic inactivation. The incomplete data on the estrone treatment of rats of these three strains, however, indicate a response similar to diethylstilbestrol. The observations recorded here clearly indicate constitutional differences in response to diethylstilbestrol and in the incidence of mammary gland and bladder cancer in the rat. That these variations are dependent upon genetically determined physiological differences in the absorption, utilization or excretion of diethylstilbestrol, rather than gene differences specifically related to cancer susceptibility, is also indicated.

SUMMARY

1. Pellets of compressed cystalline diethylstilbestrol weighing 15 to 25 mgm. were implanted in the scapular region of 30 rats of both sexes of each of 3 distinct inbred lines and were replaced individually as soon as the previous pellet was known to be completely absorbed.

2. The rats of the 3 lines varied in survival and in the absorption rate of the diethylstilbestrol. Rats of Fischer line 344 succumbed first after the most rapid absorption of the hormone. Females of this line lived an average of only 67 days and absorbed an average of 19 mgm. per rat. The males survived an average of 136 days and absorbed 29 mgm. of diethylstilbestrol per rat. The Copenhagen line 2331 males survived for 289 days and the females for 200 days, having absorbed an average of 38 and 30 mgm. of hormone, respectively. The rats of A × C line 9935 were intermediate; the males survived for 167 days and the females for 186 days, in which time the average absorption of diethylstilbestrol was 24 and 25 mgm. per rat.

3. The most conspicuous pathological lesions included pituitary adenomas, fatty livers, and bladder calculi and papillomas. Pronounced strain differences were observed in the expression of these lesions. The pituitaries in the Fischer rats averaged 300 and 400 mgm. respectively in males and females, and only 98 and 61 mgm. in Copenhagen males and females, and were intermediate in the A × C rats. Fatty livers were confined to Fischer rats, and with 3 exceptions, the bladder calculi and papillomas were confined to Copenhagen male rats.

4. Pellets of 75 per cent cholestrol containing 4 to 15 mgm. of diethylstilbestrol were implanted in the scapular region of a second and similar series of

rats and no reimplantations were necessary to keep the rats in a constant state of hyperestrinism for the remainder of their lives.

5. The average survival period for the Fischer line 344 rats was increased to 200 days for the females and 367 days for the males with the more slowly absorbed diethylstilbestrol. The A × C males lived an average of 388 days and the females 442 days whereas the Copenhagen males and females survived 528 and 499 days, respectively.

6. Mammary cancer was observed in 17 or 80 per cent of the A × C males, in 22 or 85 per cent of the A × C females. Only 1 or 17 per cent of the Fischer line 344 females and 5 or 22 per cent of the Fischer line 344 males had mammary cancer.

7. No mammary cancers were observed in treated male or female Copenhagen line 2331 rats but 16 or 62 per cent of the males and 6 or 29 per cent of the females had either bladder papillomas or squamous cell cancer associated with urinary calculi.

8. The mammary cancers induced in Fischer line 344 and A ×C line 9935 rats by slowly absorbed diethylstilbestrol included 111 adenocarcinomas, 11 adenocarcinomas and squamous cell cancers or adenoacanthomas, 4 solid carcinomas, 2 solid carcinomas with papillary areas, 1 interductal carcinoma and 4 that were unclassified.

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Partial Separation of the Mammary Tumor Milk Agent and a Comparison of Various Sources of the Agent*

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The spontaneous development of mammary tumors in mice is dependent mainly upon three factors (3): genetic susceptibility, hormones, and the milk agent, which the young obtain from their mothers during nursing.

The milk agent has been shown to pass through bacterial filters (1, 3, 4), to be destroyed at 60° C. (1, 2), to be stable for 1 to 2 hours at pH values between 5 and 10 (2), to survive through 10 transplant generations in mice that did not originally contain the agent (7), and to survive (or proliferate) when carried as a cell-free filtrate through 9 passages in eggs (7, 10). Furthermore, it has been shown that it can be sedimented from extracts of mammary glands at 110,000 times gravity (20) and from mouse milk at 60,000 times gravity (11). In these experiments the agent was assayed in test animals by injecting or feeding fractions obtained from 0.2 cc. of milk or from 0.33 to 1 gm. of mammary tumor tissue or lactating glands. More recent work (8) has shown that the agent can be demonstrated in an extract of lactating mammary glands representing 2×10^{-4} gm. of gland tissue. The presence of the agent has also been demonstrated in whole blood, spleen and thymus (5), and in liver (10).

The present work was undertaken in an effort to study more quantitatively the cytoplasmic distribution of the agent by combining the technics of differential centrifugation with those of serial dilution, and also, to attempt to find the most potent source of the agent.

MATERIALS AND METHODS

The test animals used were ABC females between 3 and 6 weeks of age obtained as follows:
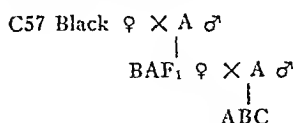
C57 Black ♀ × A ♂
|
BAF₁ ♀ × A ♂
|
ABC

The tumors or lactating glands used as sources of the agent were ground in a mortar with distilled water or saline-buffer (0.01 M phosphate at pH 7.6 in 0.85 per cent NaCl) and following centrifugation the various fractions were resuspended in saline-buffer and injected intraperitoneally or subcutaneously. The fractions were injected in amounts ranging from 10^{-2} to 10^{-7} gm. equivalents; that is, the amount of a given fraction that was derived from 10^{-5} gm. of original wet tissue is referred to as 10^{-5} gm. equivalents. Following injection the animals were force-bred for at least 2 or 3 litters and then were allowed to breed normally. The animals average 4 or 5 litters apiece. The experiments were terminated when the animals were 2 years of age and the surviving mice were autopsied. With only one exception, for all of the experiments reported in this paper the tumors were established as mammary gland carcinoma by microscopic examination.[1]

In all experiments, particularly the fractionation experiments in which preparation required up to 8 hours, the utmost care was taken to maintain the temperature of the material as close to 0° C. as possible. The tissues were harvested into a beaker in an ice-water bath, macerated by forcing through a tissue press with holes about 1 mm. in diameter, ground in a cold mortar in an ice-water bath, and centrifuged in the cold. The initial centrifugation was carried out in a Sorvall angle centrifuge operated in the refrigerator. The next two centrifugations were done in an International centrifuge equipped with a multispeed attachment. This centrifuge, which develops a force of 23,000 × g. at the center of the 14 cc. plastic tubes that were used, was operated in a cold room at about −15° C., and the material was kept just above its freezing point by adjusting the cover of the centrifuge. The final centrifugation was carried out at 70,000

[1] The authors are indebted to Dr. R. A. Huseby and Mr. Norman Kretchmer for their help in diagnosing the tumors.

× g. in an air-driven ultra-centrifuge.[2] The head of this centrifuge was chilled prior to running, and since it operates in a vacuum, the material remained quite cool throughout the run. The final fractions were always diluted in cold saline-buffer just prior to injection, and for a given fraction the most dilute solution was always injected first and the most concentrated one last. The longest time interval between removal of the tissue and injection of a fraction was in the first fractionation experiment, in which 10 hours elapsed. This particular fraction gave a good incidence of tumors.

RESULTS

In this series of experiments the first attempt to use dilute solutions for testing the activity of the agent was one in which spontaneous tumors arising in A strain mice were used as the source material. The tumors were macerated and ground in a mortar with sand and 9 volumes of distilled water. The extract was cleared of debris by spinning at about 500 × g. for 20 minutes, and the supernatant fluid was removed from under the small amount of fat on the surface. The results of injecting this supernatant fluid into test animals in 10^{-1} and 10^{-3} gm. equivalent amounts are shown in Table I.

Table I: Activity of the Milk Agent in Extracts of Spontaneous Tumor Tissue

Gram. equiv. injected	Mice living to 1st tumor	No. of mice with tumors	% with tumors	Av. tumor age, days
10^{-1}	15	12	80	307
10^{-3}	15	11	73	326

Two large scale experiments were set up involving a combination of serial dilution and fractionation by differential centrifugation. The first of these was designed largely upon the centrifugation technic described by Claude (12, 13). Spontaneous tumors arising in A strain mice were put through the tissue press and ground in a cold mortar with the slow addition of 4 volumes of cold saline-buffer. The resulting suspension was centrifuged in the refrigerator at 500 × g. for 5 minutes and the supernatant fluid decanted and spun again for 5 minutes at this speed. This supernatant fluid, the cytoplasmic extract, was labelled E. Part of the extract was spun at 23,000 × g. for 5 minutes. The resulting sediments were resuspended in saline-buffer, cleared at low speed for 1 minute, and the supernatant fluid again spun for 5 minutes at 23,000 × g. Once again the sediments were resuspended, cleared at low speed and

resedimented at 23,000 × g. for 5 minutes. This time the sediments were resuspended and referred to as the large granule, or L, fraction.

The supernatant fluid from the first 5 minute run at 23,000 × g. was spun for 1 hour at 23,000 × g., and the resulting supernate, referred to as MS, was carefully removed with a syringe and needle from beneath the few fatty particles on the surface. The pellets were resuspended in saline-buffer and cleared of large aggregates by spinning for 5 minutes at 23,000 × g. A very large sediment appeared (*see* Discussion) but this was discarded and the slightly opalescent supernatant fluid was spun again for 1 hour at 23,000 × g. The pellets were again resuspended and this time spun for 1 minute at low speed and the supernate referred to as the microsome, or M, fraction.

The four fractions available for injection were E, L, M and MS. These were diluted just prior to injection into test animals. The results are shown in Table II.

Table II: Activity of the Milk Agent in Centrifugal Fractions of Spontaneous Mammary Carcinoma

Fraction	Gram. equiv. injected	Mice living to 1st tumor	No. of mice with tumors	Av. tumor age, days
E	10^{-6}	6	0	
	10^{-5}	8	0	
	10^{-4}	8	2	330
	10^{-3}	9	4	336
	10^{-2}	10	5	319
L	10^{-7}	9	0	
	10^{-6}	9	0	
	10^{-5}	7	0	
	10^{-4}	10	0	
	10^{-3}	9	3	417
	10^{-2}	8	3	389
M	10^{-7}	8	0	
	10^{-6}	7	0	
	10^{-5}	9	1	473
	10^{-4}	6	2	400
	10^{-3}	9	1	524
	10^{-2}	8	5	327
MS	10^{-6}	8	0	
	10^{-5}	9	0	
	10^{-4}	8	0	
	10^{-3}	10	0	
	10^{-2}	8	1	440

By pooling the 3 most concentrated dilutions of each fraction, it is possible to get larger and more significant groups as seen in Table III.

Table III: Summary of Results with the 3 Most Concentrated Dilution of Each Fraction

Fraction	Gram. equiv. injected	Mice living to 1st tumor	No. mice with tumors	% with tumors	Av. tumor age, days
E	10^{-2}–10^{-4}	27	11	41	327
L	10^{-2}–10^{-4}	27	6	22	403
M	10^{-2}–10^{-4}	23	8	35	370
MS	10^{-2}–10^{-4}	26	1	4	440

The total nitrogen found in the amount of each fraction derived from 1 gm. of tumor tissue is indicated in Table IV.

TABLE IV: TOTAL NITROGEN VALUES FOR EACH FRACTION

Fraction	Mgm. N per gm. tissue
E	11.15
L	0.19
M	0.083
MS	9.05

In addition to the nitrogen determinations on these fractions, an attempt was made to estimate the ribose content, and it was evident that a large portion of the cytoplasmic ribose was still left up in the MS fraction. In an attempt to sediment some of this ribose-containing material, another experiment was set up in which 3 particulate fractions were isolated.

The technic was essentially the same as in the preceeding experiment. Tissue from spontaneous tumors in mice of the A strain was ground with saline-buffer, cleared of debris and the supernatant fluid labelled E. The L fraction was prepared as before, except that in place of the second low speed clearing run, the resuspended sediment was placed in a graduated cylinder and the heavy aggregates allowed to settle. The homogeneous supernate was carefully removed and labelled L.

In the case of the M fraction, the pellet was spun down from the supernatant fluid occurring after the first 5 minute spin at 23,000 × g., by spinning 1 hour at 23,000 × g. This pellet was resuspended, the heavy aggregates allowed to settle, and the opalescent dispersion carefully removed and labelled M.

The supernatant fluid after the 1 hour spin at 23,000 × g. was spun in the ultracentrifuge for 1 hour at 70,000 × g. The clear supernate was carefully removed from beneath the fat flakes on the surface and labelled S. The pellets were resuspended in saline-buffer, the heavy aggregates allowed to settle and the opalescent supernatant fluid removed and labelled U.

The results of injecting these 5 fractions are shown in Table V.

As may be seen, the U and S fractions produced a good incidence of tumors when 10^{-2} gram equivalents were injected, but very few tumors occurred from the higher dilutions. In comparing the fractions, it was felt most significant to pool the data from the middle 3 dilutions as shown in Table VI.

The analyses for nitrogen on these fractions are shown in Table VII.

At about the same time that these two fractionation experiments were made, we were interested in

TABLE V: ACTIVITY OF THE MILK AGENT IN CENTRIFUGAL FRACTIONS OF SPONTANEOUS MAMMARY CARCINOMA

Fraction	Gram. equiv. injected	Mice living to 1st tumor	No. of mice with tumors	Av. tumor age, days
E	10^{-6}	7	1	354
	10^{-5}	8	1	438
	10^{-4}	9	4	271
	10^{-3}	8	4	305
	10^{-2}	6	6	310
L	10^{-6}	7	0	
	10^{-5}	8	3	520
	10^{-4}	7	4	426
	10^{-3}	3	3	328
	10^{-2}	6	2	270
M	10^{-6}	5	0	
	10^{-5}	6	2	428
	10^{-4}	8	5	322
	10^{-3}	9	8	310
	10^{-2}	7	5	330
U	10^{-6}	7	0	
	10^{-5}	8	1	563
	10^{-4}	9	0	
	10^{-3}	5	2	550
	10^{-2}	9	5	417
S	10^{-6}	8	0	
	10^{-5}	8	1	618
	10^{-4}	5	0	
	10^{-3}	6	0	
	10^{-2}	6	5	333

TABLE VI: SUMMARY OF RESULTS OF TESTS OF ACTIVITY OF FRACTURES FROM POOLED DATA FOR MIDDLE 3 DILUTIONS

Fraction	Gram. equiv. injected	Mice living to 1st tumor	No. mice with tumors	% with tumors	Av. tumor age, days
E	10^{-3}–10^{-5}	25	9	36	305
L	10^{-3}–10^{-5}	18	10	56	425
M	10^{-3}–10^{-5}	23	15	65	330
U	10^{-3}–10^{-5}	22	3	14	554
S	10^{-3}–10^{-5}	19	1	5	618

TABLE VII: NITROGEN VALUES OF FRACTIONS

Fraction	Mgm. N per gm. tissue
E	11.43
L	0.27
M	0.79
U	0.90
S	8.03

attempting to discover the best source material for isolating the agent. An experiment was arranged to compare transplanted tumor tissue, spontaneous tumor tissue, and lactating glands from animals containing the agent. The transplanted tissue used was derived from a spontaneous tumor arising in an A strain mouse and was carried for two generations in A strain mice. The spontaneous tumor tissue used was also from an A strain mouse. One set of lactating glands was obtained from an A strain mouse that was 7 months of age

and was nursing her second litter which had been born 17 days before. These glands contained a fairly large amount of milk despite the fact that the young had been left with the mother until the time of sacrifice. A second set of glands was obtained from a C3H or Z mouse that was 6 months of age and was nursing her second litter born 18 days before. Here again the young were left with the mother until the time of sacrifice, but in this case the glands contained no appreciable amount of milk.

The 4 tissues were ground separately in mortars with sand and saline-buffer and then cleared by spinning 6 minutes at 500 × g. The extracts were labelled T, S, A and Z from transplanted tumor tissue, spontaneous tumor tissue, A strain glands and Z strain glands respectively.

These extracts were diluted and injected into ABC mice as in the previous experiments. In the case of the extracts from the transplanted tumor tissue, the dilutions were injected either intraperitoneally or subcutaneously, but since there was no significant difference, the results are pooled. All other injections were given intraperitoneally. In a few of the mice injected subcutaneously with extracts of the transplanted tumor tissue, there occurred transplanted tumors at the site of the injection within a few weeks, so these animals were discarded. It is apparent that a few intact cells were included in the supernate decanted after spinning 6 minutes at 500 × g. The present practice in this laboratory is to spin 4 minutes at 1500 × g. This brings down all intact cells and all or most of the nuclei. The extracts from some tissues must be spun again to clear the remaining nuclei.

The results of injecting these extracts of tumors and mammary glands are shown in Table VIII.

Here it seemed most significant to pool the data from the 3 highest dilutions as has been done in Table IX.

DISCUSSION

In analyzing the data reported here, and in attempting to correlate it with previous work in this and other laboratories two important facts stand out. The first is that in fractionation experiments based on injecting relatively concentrated preparations of the various fractions, the interpretation may be very misleading since 10^{-6} gm. of tissue have been shown to contain enough agent to induce tumors. This is brought out very clearly in Table V where the U and S fractions showed a good incidence of tumors when 10^{-2} gm. equivalents were administered, but gave rise to only occasional tumors which arose much later in life when smaller amounts were used. Had 10^{-2} gm.

TABLE VIII: Activity of the Milk Agent in Extracts of Various Tissues

Fraction	Gram. equiv. injected	Mice living to 1st tumor	No. of mice with tumors	Av. tumor age, days
T	10^{-6}	12	1	371
	10^{-5}	15	3	507
	10^{-4}	13	2	327
	10^{-3}	10	1	184
	10^{-2}	8	4	351
S	10^{-6}	6	2	395
	10^{-5}	6	2	297
	10^{-4}	8	3	378
	10^{-3}	7	5	319
	10^{-2}	8	7	294
A	10^{-6}	7	3	294
	10^{-5}	6	6	293
	10^{-4}	8	8	349
	10^{-3}	6	4	314
	10^{-2}	6	3	330
Z	10^{-6}	6	1	352
	10^{-5}	7	3	329
	10^{-4}	8	5	364
	10^{-3}	6	5	318
	10^{-2}	6	6	352

TABLE IX: Summary of Results from Pooled Data of the Three Highest Dilution of Fractions

Fraction	Gram. equiv. injected	Mice living to 1st tumor	No. mice with tumors	% with tumors	Av. tumor age, days
T	$10^{-4}-10^{-6}$	40	6	15	424
S	$10^{-4}-10^{-6}$	20	7	35	360
A	$10^{-4}-10^{-6}$	21	17	81	319
Z	$10^{-4}-10^{-6}$	21	9	43	351

equivalents been the only solution injected, the interpretation might well have been that the agent was equally distributed throughout the cytoplasm, that is, that L, M, U and S all showed the same activity. The interpretation may be even more misleading when one considers that in some instances dilution of an extract up to a certain point may actually give rise to a higher incidence of tumors (8).

A previous fractionation experiment, the preliminary results of which have been reported (2), further illustrates the difficulty of interpreting data when concentrated solutions are used. The final data on this experiment have not altered the previous conclusions, and show that when an extract of spontaneous tumor tissue was brought to a pH of 5.5 the resulting precipitate and supernatant fluid gave rise to roughly the same incidence of tumors when injected in 1 gm. equivalent amounts into small groups of test animals. Also, in this same experiment extracts of the tumor tissue were treated with the basic protein, salmine, and again there did not appear to be a very clear-cut separation of the agent as determined by in-

jecting 1 gm. equivalents of precipitate and supernatant fluid into test animals. Certainly part of the difficulty in interpreting such a fractionation experiment is due to the tremendous doses used, and it is possible that serial dilution might have shown one fraction to be much more active than another. This is unlikely in this particular fractionation experiment, however, since the major portion of the agent is now known to be associated with the large granule and microsome fractions of the tumor cell cytoplasm as seen in Tables II and V. Claude (13) has shown that these fractions from liver cells are maximally precipitated at a pH of 3.5, but that precipitation is not complete at pH 5.5. Furthermore, unpublished work in this laboratory has shown that the concentration of salmine used in the above experiment (0.1 per cent) precipitates only about 30 per cent of the microsome fraction from a cytoplasmic extract of tumor tissue, though the same concentration of salmine will almost completely precipitate a suspension of purified microsomes. It would seem probable, therefore, that the agent was not separated in any clear cut manner either by 0.1 per cent salmine or at pH 5.5; but such a conclusion would not be justified from the data on the incidence of tumors, even though this incidence were identical in two fractions, since only one concentration of each fraction was injected.

A second observation that seems important for future work has been that when transplanted tumor tissue was used as the source of the agent, the results were extremely erratic. In one experiment, for example, an extract of transplanted tumor tissue was injected into 130 mice in amounts ranging from 10^{-2} to 10^{-6} gm. equivalents. Only 25 of these mice developed tumors, but the surprising fact was that these tumors were rather equally distributed among all animals that received diluted material in the series. A comparable situation is seen in Tables VIII and IX, where the transplanted tumor tissue has given rise to much the lowest incidence of tumors but still there are tumors occurring after injection of 10^{-5} and 10^{-6} gm. equivalents of the materials tested. No adequate explanation is apparent for this behavior, but it does indicate that transplanted tumor tissue is a much less reliable source of the agent for experimental work than are, for example, mammary glands.

From the data reported in Tables VIII and IX it is apparent that the lactating glands of the A strain mouse used in this experiment were an entremely potent source of the agent. The dilutions must be carried further since 10^{-6} gm. equivalents still gave rise to a significant incidence

of tumors. It must be pointed out that these were glands from a single mouse, as was true also for the C3H or Z gland tissue used in this experiment, and consequently the somewhat lower incidence of tumors arising from the extract of the Z glands cannot be definitely interpreted as a strain difference but may be merely an individual difference.

The available history on the A strain mouse whose glands were used shows that the mother and one of two daughters died of mammary cancer. The second daughter was an experimental animal that had been placed on a calorie-restricted diet for which the incidence of cancer was very low. In the case of the Z strain mouse the mother and 3 of 4 sisters died of mammary cancer. This possibility of a strain difference should be tested further at higher dilutions from extracts obtained from the pooled glands of many mice of the two strains. In comparing the relative activity of the milk agent in the glands of A and Z strain mice, the test animal should also be taken into consideration. It is possible, for example, that the agent from glands of Z strain mice might show a greater activity if tested in ZBC mice. However, it is interesting to note that these present results, which indicate possibly a greater activity of the agent from A strain mice, are in accord with the findings on reciprocal crosses of A and Z mice (6, 9). In this work the first and second generation hybrids with A mothers and Z fathers (AZF_1 and AZF_2) showed a significantly higher incidence of tumors than the reciprocal crosses (ZAF_1 and ZAF_2) that had Z mothers. In as much as the genetic constitution of the two crosses should be identical, it seemed most plausible to ascribe the differences in incidence to differences in the concentration and/or activity of the milk agent in the milk of the A and Z mothers.

Since the agent is extremely potent in lactating mammary glands but apparently much less potent in blood (10), it seems unnecessary to postulate that its occurrence in milk may be due to the presence of lymphoid elements containing the agent as suggested by Gardner (14, 15).

As seen in Tables VIII and IX, the transplanted tumor tissue used appeared to be the least potent source of the agent. Again this tissue was derived from a single spontaneous tumor that was carried for two generations in A strain mice, that is, in mice who themselves contained the agent. However, the agent has been demonstrated (7) in transplanted tumor tissue carried for 10 generations in mice that did not themselves contain the agent.

The tissue from spontaneous tumors that was used in this experiment and in the two fractiona-

tion experiments appeared to be a fairly good source of the agent though probably not as good as lactating glands from mice of the A stock. Again it should be pointed out that, while the present work requires extension, the indication that it gives of a greater activity of the agent in lactating glands of the A strain mouse was obtained only because dilutions were carried out to 10^{-6} gm. equivalents. Had these dilutions been only from 10^{-2} to 10^{-4} gm. equivalents then, as seen from Table VIII, the incidence of tumors would have been as great from spontaneous tumor tissue and from glands of the Z strain mouse as from the glands of the A strain mouse.

The fractionation experiments shown in Tables II, III, V and VI indicate that only a very small fraction of the agent, probably less than 1 per cent, remains in the supernatant fluid after spinning extracts of spontaneous tumor tissue at 23,000 × g. for 1 hour. The differences between the two larger particulate fractions, the large granules and the microsomes, are not nearly so clear-cut. There is a slight indication, based largely on average tumor age, that the agent may be more active in the microsome fraction, but this is by no means certain and will require further work.

The large granule and microsome fractions present a very distinct difference in appearance in the sedimented states. The large granules form a fairly well-packed, tan, opaque sediment, while the microsomes give a tightly packed, light-amber pellet that is perfectly transparent except for a very small, whitish star at the bottom. With improvement of technics it is now possible to resuspend both of these fractions to such a state that a smaller portion is lost when they are cleared at lower speeds. However, subsequent work with liver and with tumor tissue has led us to the conclusion that it is not necessary to clear the resuspended microsomes, for example, by a 5 minute spin at 23,000 × g., since it appears that all of the large granules (and probably some microsomes) are brought down by the initial 5 minute spin at 23,000 × g. As seen by comparing Tables IV and VII, the clearing run in the first experiment brought about the loss of about 90 per cent of the microsome fraction. The present practice in this laboratory, therefore, is to resuspend and then resediment these fractions without employing any low speed clearing runs.

In the two fractionation experiments Bial's orcinol-HCl test (17) was employed in an attempt to quantitate the ribose in the various fractions. The ribose, as found by this test, in an MS fraction was approximately equally distributed between the U and S fractions. Subsequent work, employing the Beckman ultraviolet spectrophotometer and the technics of Schneider for separation of nucleic acids (18) has shown that the major portion of the ribose of the particulate fractions (L, M and U) is present as nucleic acid. This is true whether these fractions are derived from liver, tumor tissue, mammary glands containing the agent, or mammary glands without the agent. Consequently, the finding of nucleic acid in a fraction that contains the agent cannot be cited as evidence that the agent is a nucleoprotein complex, as Shimkin (19) has interpreted the results obtained by Kahler and Bryan (16).

SUMMARY

By employing the technics of serial dilution, A strain lactating mammary glands have been shown to be an extremely potent source of the mammary tumor milk agent. Extracts give rise to tumors even at a million-fold dilution. C3H or Z strain lactating glands and spontaneous tumor tissue appear to be a less potent source of the agent, while transplanted tumor tissue seems to be a relatively poor and unreliable source.

The agent can be essentially completely sedimented from an extract of spontaneous tumor tissue by centrifuging for 1 hour at 23,000 × g. The agent has been found in both the large granule ("mitochondria") fraction and in the microsome fraction of a cytoplasmic extract of spontaneous tumor tissue.

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The Response of Mice to the Simultaneous Application of Two Different Carcinogenic Agents

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In a previous paper we reported on results of experiments in which rats were treated with two differently acting carcinogens (2). That study was made in order to investigate the possible influence of one such agent on the action of another. No interaction between the carcinogens was found. The present report deals with similar experiments on albino mice.

MATERIALS AND METHODS

Animals used in these experiments were albino mice of our own breed, 6 to 8 weeks old. The spontaneous tumor rate in this stock is 0.5 per cent at the age of 1 year if lung tumors are not included. The latter have been observed in 5 per cent of the animals autopsied at the age of 4 months, in 15 per cent of mice 6 months old and in 25 per cent of mice at 1 year of age.

The methylcholanthrene was dissolved in 0.1 ml. of olive oil and injected subcutaneously. A single injection was given to each animal. Urethane and *p*-dimethylaminoazobenzene were mixed with the diets in the amounts indicated in Table I and in the same manner as previously described (2). The carcinogenic diet was given from the day of injection of methylcholanthrene through the whole experimental period. At the end of 4 months the animals were killed and autopsied and examined for local tumors. The lungs were fixed in Bouin solution and formol as previously described (3) and examined for the presence of adenomas.

RESULTS

In Table I, a summary of the animals with local tumors and with pulmonary adenomas is given. In no case was a significant difference observed between the tumor incidence in the groups treated with 2 carcinogens as compared with the control groups treated with only 1 of the carcinogens.

The treatment with *p*-dimethylaminoazobenzene never caused detectable liver damage under the experimental conditions. Nevertheless, the dye, when fed with the diet, can be considered to

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TABLE I: LOCAL AND LUNG TUMORS OBSERVED IN MICE 4 MONTHS AFTER BEGINNING OF TREATMENT

Treatment	Effective total	Local tumors %	Lung tumors, %
0.9 mgm. MC* subcu. + diet cont'g. 0.75% ethyl urethane	28	87	89
0.45 mgm. MC subcu. + diet cont'g. 0.15% ethyl urethane	75	76	95
0.225 mgm. MC subcu. + diet cont'g. 0.15% ethyl urethane	27	46	96
0.45 mgm. MC subcu. + diet cont'g. 0.015% *p*-dimethylaminoazobenzene	31	74	23
0.225 mgm. MC subcu. + diet cont'g. 0.015% *p*-dimethylaminoazobenzene	23	39	43
CONTROLS			
0.9 mgm. MC subcu.	164	80	45
0.45 mgm. MC subcu.	78	67	32
0.225 mgm. subcu.	68	41	31
Diet cont'g. 0.75% ethyl urethane	50	—	98
Diet cont'g. 0.15% ethyl urethane	14	—	100
Diet cont'g. 0.015% *p*-dimethylaminoazobenzene	22	—	25

* MC = methylcholanthrene

be carcinogenic for the strain of mice used, for the data in Table II show that a significant rise in the rate of pulmonary adenomas over the spontaneous rate can be observed after 75 days of feeding the carcinogenic dye. Andervont, White and Edwards (1) have shown that *o*-aminoazotoluene produces lung tumors when fed to mice, but they did not observe such an effect with *p*-dimethylaminoazobenzene in their experiments with strain C mice.

TABLE II: LUNG TUMORS OBSERVED IN MICE 75 DAYS AFTER BEGINNING OF FEEDING ETHYL URETHANE OR *p*-DIMETHYLAMINOAZOBENZENE

Treatment	Effective total	Lung tumors, %
Diet cont'g. 0.015% *p*-dimethylaminoazobenzene	50	22
Diet cont'g. 0.15% ethyl urethane	75	59
Controls	57	5

DISCUSSION

The results of experiments with mice confirm those obtained previously with rats (2), namely that *p*-dimethylaminoazobenzene or ethyl urethane do not influence the number of tumors elicited by methylcholanthrene. The number of mice developing local tumors after a subcutaneous injection of methylcholanthrene was the same whether the animals received simutaneously another carcinogen with the diet or whether they were kept on the stock diet without further treatment.

Under the experimental conditions it was not possible to decide whether the action of injected methylcholanthrene in producing pulmonary adenomas in mice is additive to the action of urethane as the number of animals developing this kind of tumor was close to 100 per cent. In 2 series treated with methylcholanthrene and fed a diet containing *p*-dimethylaminoazobenzene no such additive action could be detected but the number of mice was not sufficient to draw definite conclusions.

The results indicate that in general 2 different carcinogens act independently. This observation does not necessarily exclude the possibility that under special conditions an interaction may be observed. It is known that carcinogenic *hydro*carbons may have a retarding action on growth of transplanted tumors (7) and recently Murphy and Sturm (6) found that ethyl urethane inhibits the development of lymphatic tumors and leukemia in rats. This compound has a definite carcinogenic action in rats (4).

Indeed, we found recently that mice that had received *p*-dimethylaminoazobenzene with the diet for 6 weeks previous to a single injection of urethane developed fewer pulmonary adenomas than did the controls (5). Therefore care should be taken not to accept the independent action of simultaneously applied differently acting carcinogens as a rule without having studied each special case.

The present experiments show that feeding *p*-dimethylaminoazobenzene at a level of only 0.015 per cent for 75 days raises the spontaneous rate of pulmonary adenomas to a significant degree. No such action of the azo dye has been reported previously as far as we know, although the related compound, *o*-aminoazobenzene, has been shown to stimulate the development of pulmonary tumors in mice (1).

SUMMARY

Albino mice were injected subcutaneously with methylcholanthrene and were given a diet containing sufficient ethyl urethrane to produce pulmonary adenomas in nearly 100 per cent of the animals during the experimental period of 4 months. No difference in the number of animals developing local tumors was observed as compared with the controls. *p*-Dimethylaminoazobenzene fed in the diet also did not influence the number of local tumors elicited by methylcholanthrene.

Feeding of a diet containing *p*-dimethylaminoazobenzene alone augmented the number of mice bearing pulmonary adenomas to a significant degree over the spontaneous rate, while no liver damage could be detected under the conditions observed.

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Studies On Drug Adsorption

Fixation of Quinine by Neoplastic and Non-Neoplastic Tissues*

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The observations reported in this paper were made in patients with a variety of malignant neoplasms. The purpose was to determine whether quinine might be selectively concentrated in such neoplasms. Previous studies on laboratory animals (1-3) revealed a striking species variation in quinine metabolism as well as an uneven distribution of quinine in the tissues and fluids of the body.

Many of the investigations on the biochemical variations between neoplastic and non-neoplastic tissues have concerned themselves with a direct analysis of tissue components and enzyme activities. It was hoped that the more indirect approach of observing the degree of concentration of drugs in such tissues might reveal quantitative differences that could eventually be used in designing a drug which would be specifically localized in neoplasms.

MATERIALS AND METHODS

Quinine, as the hydrochloride salt, was administered either by mouth or by vein from 2 to 7 hours preoperatively, to a selected group of patients with various types of neoplasms. Blood was drawn for analysis at the beginning and end of the operation, and the time of removal of the tumor and adjacent tissues noted. In all patients, the diagnosis was confirmed by histologic examination of the excised tissues.

Chemical procedures.—Analyses for quinine were done by a modification of a previously described method (1). Blood, plasma or tissue homogenate was dissolved by warming in 10 per cent KOH and after cooling was extracted by ethyl ether. An aliquot of the ether phase was extracted by shaking with 0.1 N H_2SO_4 and the fluorescence of the acid extract determined in a Coleman photofluorometer, model 12A, with B_1S and PC-1 filters. This method is sensitive to 0.1 μgm. Control analyses on tissues from patients who had received no drug gave values ranging from 0.1 to 0.4 μgm. of "apparent quinine" per gram of tissue.

Total nitrogen values were obtained by a micro-Kjeldahl procedure. The separation and the analyses of the acid-soluble, phospholipid and nucleic acid phosphorus fractions were done by the methods of Schneider (5).

RESULTS AND DISCUSSION

The data obtained from 29 patients are given in Tables I and II. With four exceptions, the concentration of the drug in the neoplasm was greater than in the corresponding normal tissue. The magnitude of the difference in concentration was quite variable. Values of from 1.5 to 2 times were commonly obtained and on 2 occasions the concentrations observed in breast carcinomas were more than 7 times those observed in the normal breast tissue of the same patient. Analyses of such other tissues as were available are included to emphasize the fact that distribution of this drug in the body is very uneven and that certain tissues (*e.g.*, spleen, lymph nodes and liver) characteristically contain even larger amounts than the neoplasms themselves.

There are doubtless several mechanisms at play in determining the degree of fixation of the drug in any given tissue, but it seems justifiable to conclude that there is some process occurring in neoplastic cells that increases this fixation in comparison with the "parent" tissue from which the neoplasm arose. The nature of this process is obscure. The ubiquitous role of phosphorus compounds in tissue metabolism suggested the analyses recorded in Table II. It can be seen that there is no obvious correlation between the quinine concentration and either the total nitrogen or the phosphorus values obtained on neoplastic and normal tissues.

That the process is not related to simple tissue damage or degeneration is shown by the lower concentrations observed in the involved areas of the mucosa and muscle of the patient with regional ileitis and in the cystic area of the breast in the patient with non-malignant diseased breast.

SUMMARY

Analyses were made of the extent of quinine localization in a variety of human malignant neoplasms and corresponding normal tissues. In most cases, a higher concentration was observed in neoplastic tissue than in the tissue of origin. However, the concentration observed in liver, lymph nodes, spleen and certain other tissues was much higher than that observed in the neoplasms. No correlation was observed between the quinine concentration and the nitrogen or phosphorus content.

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TABLE I: CONCENTRATION OF QUININE IN NEOPLASTIC AND NON-NEOPLASTIC TISSUES AFTER ORAL INTRAVENOUS ADMINISTRATION OF QUININE

Patient No.	Diagnosis	Route*	Time after drug Hr:Min.	Plasma** mgm./l	Carcinoma mgm./kgm.	Other tissues mgm./kgm.
25	Carcinoma, skin of orbit	I.V.	2:30	6.6	17.2	Skin, 12.7; muscle, 8.3
2	Carcinoma, tongue	Oral	4:10	3.5	15.6	Mucosa, 9.0; muscle, 9.0
28	Carcinoma, larynx	I.V.	2:30	3.5	5.2	Mucosa, 4.0; muscle, 3.8
39	Carcinoma, larynx	I.V.	3:00	3.5	10.5	Mucosa, 6.2; muscle, 4.8
1	Recurrent carcinoma, neck (Primary, larynx)	Oral	3:15	4.8	18.2	Skin, 11.8; muscle, 8.3; lymph nodes, 15.7; carotid artery, 7.6
18	Carcinoma, esophagus	I.V.	3:10	4.3	13.4	Esophageal mucosa, 10.0; gastric mucosa, 11.7; spleen, 46.3
33	Carcinoma, esophagus	I.V.	3:30	6.5	17.0	Mucosa, 12.0; muscle, 8.0; lymph nodes, 24.0
9	Carcinoma, stomach	I.V.	4:20	6.8	17.2	Gastric mucosa, 12.0; gastric muscle, 6.6; colon mucosa, 8.6; colon muscle, 6.7
27	Carcinoma, stomach	I.V.	2:00	5.0	19.0	Mucosa, 12.5; spleen, 24.0
29	Carcinoma, stomach	I.V.	3:00	8.7	15.3	Mucosa, 8.2; liver, 40.0; spleen, 18.0
37	Carcinoma, stomach	I.V.	4:00	7.0	12.3	Mucosa, 10.1
3	Carcinoma, colon	Oral	4:10	4.6	31.3	Mucosa, 15.5; muscle, 6.0; peritoneum, 2.2; anal skin, 14.3
13	Carcinoma, colon	I.V.	3:15	6.5	12.4	Colon mucosa, 7.9; ileal mucosa, 14.4; liver, 42.7
20	Carcinoma, colon	I.V.	3:30	4.6	30.5	Mucosa, 17.6; muscle, 6.7
30	Carcinoma, colon	I.V.	2:30	6.2	8.3	Mucosa, 12.5; muscle, 4.0; skin, 5.1
40	Carcinoma, colon	I.V.	7:40	4.6	5.3	Mucosa, 4.6; kidney, 8.3; spleen, 8.9
34	Carcinoma, anal colon	I.V.	3:45	4.5	5.3	Skin, 6.0
36	Carcinoma, rectum	I.V.	2:20	3.4	9.9	Mucosa, 6.2; muscle, 3.4
19	Carcinomatosis	I.V.	2:20	13.9	16.4	Abdominal wall muscle, 12.9; subcutaneous fat, 4.5
21	Carcinoma, skin	Oral	5:45	8.7	9.6	Normal skin, 14.0; cicatrized skin, 9.3; synovia from knee joint, 4.6; quadriceps tendon, 3.1
32	Carcinoma, cervix	I.V.	3:30	7.2	48.5	Mucosa, 21.0; vaginal epithelium, 10.0
8	Carcinoma, breast	Oral	3:20	7.5	38.0	Pectoral muscle, 9.0; normal breast, 4.8; breast fat, 3.0; skin, 5.9; lymph nodes (involved), 16.3
23	Carcinoma, breast	Oral	4:50	5.4	10.9	Pectoral muscle, 8.5; normal breast, 8.0; skin, 6.8; lymph nodes (involved), 24.0
35	Carcinoma, breast	I.V.	3:15	2.7	11.0	Pectoral muscle, 4.5; normal breast, 1.5; skin, 2.5; lymph nodes (involved), 8.0
14	Carcinoma, head of pancreas	I.V.	2:45	2.3	10.3	Normal pancreas, 10.8; gastric mucosa, 6.9; duodenal mucosa, 5.1
22	Duodenal ulcer	I.V.	1:45	3.8	..	Pyloric mucosa, 13.2; fundus mucosa, 14.5; muscle, 6.1
7	Diseased breast (non-malignant)	Oral	3:00	3.9	..	Normal breast, 2.3; cystic breast, 1.9
5	Inflammatory "tumor," thigh	Oral	4:10	7.1	..	"Tumor," 9.4; muscle, 5.6; skin, 8.0; subcutaneous fat, 2.4
6	Regional ileitis	Oral	2:15	7.2	..	Uninvolved mucosa, 18.8; involved mucosa, 13.6; uninvolved muscle, 9.9; involved muscle, 6.6

*The dosage used was 1.0 gm. by mouth or 0.5 gm. by vein.
**Calculated from data obtained on blood drawn before and after tissues were removed.

4. Kelsey, F. E., and Brunschwig, A. Concentration of Quinine in Gastrointestinal Cancers. Preliminary Report. J. Nat. Cancer Inst. 7:355-356. 1947

5. Schneider, W. C. Phosphorus Compounds in Animal Tissues. I. Extraction and Estimation of Desoxypentose Nucleic Acid and of Pentose Nucleic Acid. J. Biol. Chem., 161:293-303. 1945.

TABLE II: Nitrogen and Phosphorus Analyses of Neoplastic and Non-Neoplastic tissues

Patient no.[*]	Site of carcinoma	Tissue	Quinine as % of plasma concentration	Total nitrogen (%)	Phospholipid phosphorus (mg%)	Nucleic acid phosphorus (mg%)	Acid soluble phosphorus (mg%)
28	Larynx	Carcinoma	150	2.5	45	45	43
		Mucosa	110	2.0	26	25	22
		Muscle	110	3.0	31	11	87
33	Esophagus	Carcinoma	260	2.9			
		Mucosa	180	2.5			
		Muscle	120	2.9			
		Lymph node	370	3.0			
27	Stomach	Carcinoma	380	2.2	56	59	
		Mucosa	250	1.6	52	64	
		Spleen	480	3.4	73	105	
29	Stomach	Carcinoma	180	2.6	70	90	88
		Mucosa	90	2.6	36	53	77
		Liver	460	3.0	83	61	101
		Spleen	210	2.9	72	86	100
37	Stomach	Carcinoma	180	2.4			
		Mucosa	140	2.3			
13	Colon	Carcinoma	190	2.0			
		Liver	660	1.9			
30	Colon	Carcinoma	130	1.9	33	78	60
		Muscle	70	1.8	52	12	63
32	Cervix	Carcinoma	670	2.5			
		Mucosa	290	1.8			
		Vaginal epithelium	140	2.0			
8	Breast	Carcinoma	510	3.2			
		Pectoral muscle	120	2.2			
35	Breast	Carcinoma	410	3.1	31	84	114
		Pectoral muscle	170	3.5	47	12	167
		Lymph nodes (involved)	300	1.3	45	48	71

[*]For further details of the results obtained on each patient, *see* Table I.

Observations on the Ketosteroid Content of Urine from Patients with Prostatic Carcinoma and Adenoma*

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The relation of sex hormones to enlargement of the prostate was reviewed by Dodds (2). Observations by Huggins and associates (5) led to the conclusion that androgens stimulate activity of the prostate and that estrogens have a depressing effect. Dean (1) reported observations on the androgen content of urine from cases of prostatic carcinoma before and after treatment by castration and by administration of estrogens. Urinary levels prior to castration appeared to be low. Data on urinary excretion of androgens have been reported by Fraser and associates (3), mainly for normal subjects and for persons with endocrine disorders. The content of 17-ketosteroids in the urine of patients with prostatic cancer was determined before and after castration by Scott and Vermeulen (6). Normal values have been given also by Venning and Kazmin (7). Since there has been a wide-spread assumption that sex hormones were involved in the production of carcinoma of the prostate, it seemed advantageous to determine urinary excretions of ketosteroids by patients having carcinomas or adenomas of the prostate.

METHODS

The quantity of 17-ketosteroids was determined chemically by a modification of the procedure of Holtorff and Koch (4), involving an adaptation of the Zimmerman color reaction (8). Briefly, the method involves hydrolysis to liberate the ketosteroids from esters, extraction of the ketosteroids with benzene, removal of phenolic material with sodium hydroxide, and the development of color by combination of ketosteroids with m-dinitro benzene in the presence of aqueous potassium hydroxide. Color determinations were made in the Coleman universal spectrophotometer set at 520 mμ., using pure androsterone as a standard. Neutral 17-ketosteroids were calculated as androsterone. Recovery of known amounts of androsterone added to urine samples averaged 94 per cent.

Except in special cases noted below, 24-hour specimens of urine were used. Usual preservatives, such as toluene and chloroform, which are solvents for ketosteroids, are unsatisfactory for this analysis. Benzoic acid was found to be suitable since it preserved the urine and did not interfere with the determination. It was employed in a concentration of 2 gm. per collection bottle (0.1 to 0.2 per cent weight by volume). Urine specimens were kept cool whenever possible, and analyses were made shortly after collection was complete.

Under prevailing conditions of staff shortages in hospitals, there was some difficulty in collecting 24-hour specimens. It was suggested that morning specimens would give sufficient information. Analyses of samples from 2 normal subjects, obtained thrice daily for 4 consecutive days, showed considerable variation in 17-ketosteroid content. Contrary to expectation, the morning specimen did not always have the highest concentration. Because of the variability found in these specimens, it seemed advantageous to use only 24-hour samples.

Observations on cases of prostatic carcinoma and adenoma.—About 200 specimens were obtained from 32 cases of carcinoma and from 39 cases of adenoma. The levels were contrasted with those found in specimens from 19 normal males. Data are given in Table I. The range of values found for normal subjects is in agreement with the observations for similar persons reported by Fraser and associates (4), by Scott and Vermeulen (6) and by Venning and Kazmin (7). It should be noted that normal subjects were appreciably younger than persons in the carcinoma and adenoma groups. It was not possible to obtain specimens from normal men of older ages. Total 24-hour excretion of ketosteroids by cases of carcinoma and adenoma of the prostate were fairly similar and were definitely lower than values found for younger normal persons. If prostatic carcinoma results from, or is coincident with, an abnormal secretion of androgenic hormones, an increased output in the urine would be expected.

This was not the case in the patients on whom observations were made. The lower values actually found were similar to the precastration data reported by Dean (1) and are probably a reflection of the ages of the subjects. They are intermediate between the values for older subjects found by Fraser and associates (3) and by Venning and Kazmin (7).

TABLE I: 24-HOUR EXCRETION OF 17-KETOSTEROIDS

	A Controls	B Adenoma of prostate	C Carcinoma of prostate
Number of subjects	19	39	32
Age in years	20-60	48-82	56-89
Range	10.1–23.2 mgm.	2.8–18.2 mgm.	2.7–16.8 mgm.
Average	16.2 mgm.	10.2 mgm.	10.0 mgm.
Standard deviation	4.02 mgm.	4.00 mgm.	4.12 mgm.
Correlation (r) with urine volume	0.4930*	0.1370	0.4732†

*Significant at the 5 per cent level.
†Significant at the 1 per cent level.

Relation of ketosteroid output and urine volume.—In considering the data shown in Table I it was observed that there was some indication of a relation of the total amount of ketosteroids to the volume of urine. Calculation of correlation coefficients showed that the relation was significant at the 5 per cent level for normal subjects and at the 1 per cent level for carcinoma patients. The relation was not significant for adenoma patients.

To obtain further information on a correlation between volume of urine and output of ketosteroids, 24-hour specimens were obtained, for 13 consecutive days, from a normal male, 45 to 50 years of age. During the first 9 days no attention was paid to ingestion of fluids and the variation in output was small. The correlation coefficient for this period was very slightly less than significant at the 5 per cent level. During the remaining 4 days, the subject took large amounts of fluids. Urinary volume and content of 17-ketosteroids were markedly increased, the latter by about 50 per cent. For this entire series of specimens, the correlation between volume and total output of 17-ketosteroids was significant at the 1 per cent level. The data are given in Table II.

Relation of ketosteroid concentration to specific gravity.—Arising from the observations on the relation between volume and the total daily excretion of ketosteroids, it seemed advantageous to determine if there was also a relation between specific gravity of urine and the concentration of ketosteroids (*i.e.*, the amount per unit of volume).

TABLE II: 17-KETOSTEROID EXCRETION OF A NORMAL MALE, 45-50 YEARS OF AGE. RELATION OF URINE VOLUME TO TOTAL EXCRETION, (mgm./24 hours)

	9 consecutive days	13 consecutive days
Range	14.6–18.7	14.6–24.0
Average	16.3	16.8
Standard deviation	1.70	2.66
Correlation (r)	0.5369	0.7669†

†Significant at the 1 per cent level.

For this series of samples from a normal subject referred to in the previous section, there was a highly significant correlation. The data are given in Table III. This correlation was not suspected until after analyses had been made on a considerable number of the hospital specimens and for these earlier samples specific gravity was not measured. Hospital specimens on which specific gravity was determined showed an indication of the same correlation, as determined by plotting specific gravity against ketosteroid concentration.

TABLE III: 17-KETOSTEROID EXCRETION OF A NORMAL MALE, 45-50 YEARS OF AGE. RELATION OF SPECIFIC GRAVITY OF URINE TO CONCENTRATION, (mgm./100 cc.)

	9 consecutive days	13 consecutive days
Range	1.18–1.84	0.88–1.84
Average	1.48	1.34
Standard deviation	0.26	0.29
Correlation (r)	0.8723†	0.9012†

†Significant at the 1 per cent level.

DISCUSSION

The data given in Table I show that in a series of 32 patients, in whom a diagnosis of prostate carcinoma was definitely made, there was not an abnormally high output of 17-ketosteroids in the urine. In fact the output was less than that observed in normal males of younger age. The amounts observed were probably compatible with the ages of the patients. The data are in agreement with the report of Scott and Vermeulen (6). It is concluded that the estimation of 17-ketosteroids in the urine is of no value as a guide to hormone therapy in the treatment of carcinoma of the prostate.

The observed relations between daily output of ketosteroids and urinary volume and between urine specific gravity and ketosteroid concentration cause doubt to arise as to the value of determinations of ketosteroids in urine for diagnostic purposes. The assumption is made frequently that estimation of a constituent of urine is an indication of the amount of the constituent in the blood and in the body. The data here recorded give an impression that the daily excretion of ketosteroids varies with the amount of urine

large cities may be laid to the general level of ignorance of the people. Beset with superstition and a belief in the efficacy of the herbs and the medications prescribed by the quacks and laymen they patronize, many die before proper medical attention can reach them. The cause of death in such cases remains unknown unless special investigation is made by medical officers of health. Such investigations are made and often do disclose the real cause of death, but many cases remain in which the true cause is never known. The picture becomes still more confused when one considers the fact that no proper control over certification is maintained so that many deaths from cancer, for example, may have been certified as due to some other cause.

The remarks contained in the paragraphs to follow must be considered in the light of the characteristics of the system of mortality reporting just described: a registration area comprising less than one-third the total population and predominantly urban in an essentially rural country, a deficiency of 50 per cent in medical certification of death in the better sections of the area, and no control over the diagnostic certification of death.

CANCER MORTALITY—GENERAL

The recorded death rate per 100,000 from cancer varied between 17.7 in 1927 and 27.6 in 1935 over the period 1918-1942. The general level of mortality may be contrasted with that for other countries in Table I, for the years 1926-1928 (12).

TABLE I: CANCER DEATH RATES PER 100,00 POPULATION FOR VARIOUS COUNTRIES, 1926-1928

Country	1926	1927	1928
Australia	93.3	92.4	94.8
Belgium	84.7	86.8	91.0
Bulgaria	——	63.0	——
Czechoslovakia	107.5	107.9	110.8
Denmark	140.0	141.0	144.0
Egypt	20.5	17.7	19.6
England and Wales	136.2	137.6	142.5
Finland	64.4	79.2	84.1
France	87.7	95.0	97.0
Germany	118.0	123.0	126.0
Greece	29.0	34.0	32.2
Japan	68.0	70.0	——
Lithuania	——	31.0	31.4
Mexico	17.0	15.7	——
New Zealand	99.1	96.3	98.8
Spain	——	68.0	70.1
Sweden	120.2	119.8	——
Switzerland	143.0	150.0	145.0
United States	94.9	95.6	96.1

With the exception of the rates for Mexico, Egypt's cancer mortality is lowest on the list. Rates for Czechoslovakia, Denmark, England,

Germany, Sweden, Switzerland, and the United States are from 5 to 7 times the rate for Egypt.

The trends of cancer mortality in the registration area of Egypt, and in the two principal cities, are shown in Fig. 1. The period prior to 1926 was one of a constant rate varying between 22 and 26. This was the period during which the registration area was limited to the 2,000,000 population of the governorates and capitals of provinces. The rate fell during the years 1926-1932, following the extension of the registration area to cover another
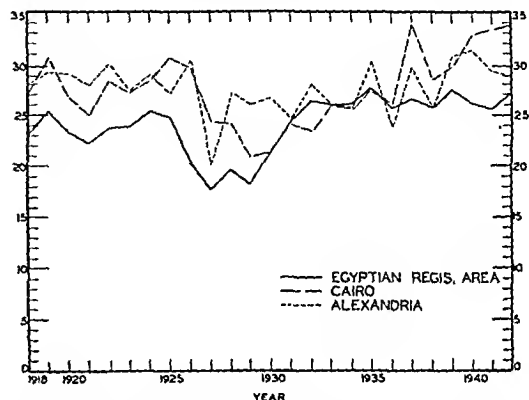
FIG. 1.—Trend of cancer mortality in Egyptian Registration Area, in Cairo and in Alexandria. Deaths per 100,000 population.

1,000,000 in the rural regions, and slowly recovered as registration gradually improved. Following 1932 the rate for the registration area has remained constant at a level slightly above that for the first period. In the two cities, however, the rate has shown a fairly steady rise, particularly in Cairo.

A regional presentation of cancer mortality is given in Fig. 2. The death rates are for the year 1942, and for the cities and towns designated. Rates for the rural sections cannot be computed because of the lack of rural population data for the registration area. The rates range from 42.6 in the governorate of Ismailia to 8.9 in that of Damietta and 4.5 in the chief town of Asswan. The wide range of rates may be ascribed in part to the relatively small population in many of the towns and governorates. The figure shows no special concentration of either high or low rates in any particular region.

DATA FROM BIOPSIES AND AUTOPSIES

Out of 20,280 specimens from biopsies and autopsies sent for examination to the pathological laboratory of the Ministry of Public Health, Cairo, from 1928 to 1937 inclusive, *3,031 or 15 per cent*
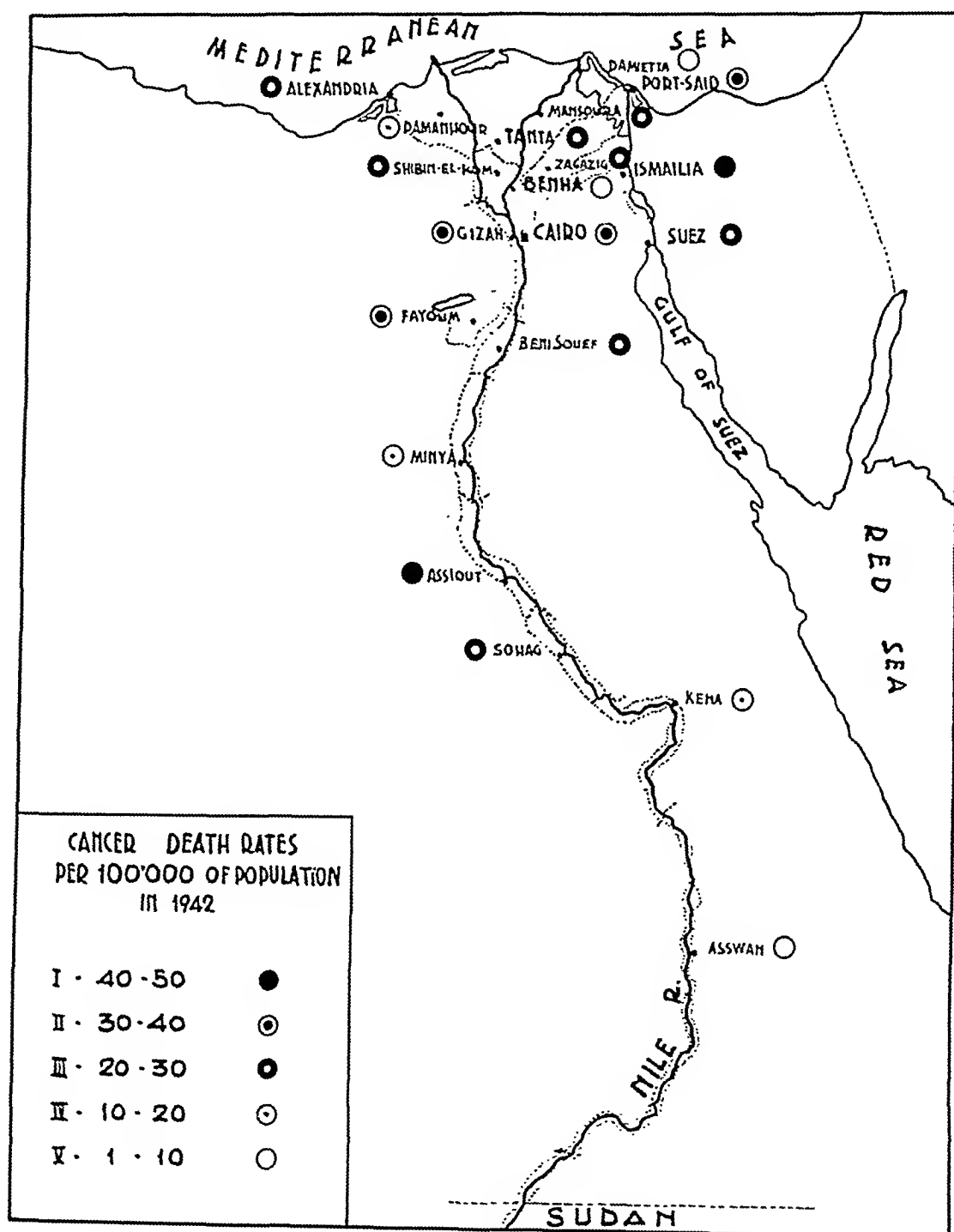
FIG. 2.—Geographical distribution of cancer mortality in Egypt, 1942.

were classified as benign and 3,180, or 15 per cent, as malignant. The remaining specimens were not tumors (7).

Of 651 specimens taken from hospital patients of the Government Hospital in Alexandria from September, 1931 to November, 1938, 330 cases (15 per cent) were classified as benign and 401 (18 per cent) as malignant. The malignant lesions were classified as carcinomas, 292 (72 per cent); sarcomas, 78 (19 per cent); endotheliomas, 9 (2 per cent); and other tumors, 22 (6 per cent) (8).

During the period 1931-1938, 880 autopsies were made in the Alexandria Government Hospital; 79 of these (9 per cent) showed malignant disease (8). In Kasr-el-Ainy Hospital in Cairo, 5,924 postmortem examinations were made during the years 1905-1930 inclusive. Malignancy was found in 365 cases, or 6.2 per cent. The cases of malignant disease were analyzed by Professor M. F. Sorour (16) and were as listed in Table II.

TABLE II: SOROUR'S ANALYSIS OF 5,924 POSTMORTEM EXAMINATIONS MADE IN PERIOD OF 1905-1930 INCLUSIVE

Site of malignant disease	Number of cases			Percentage of total
	Epithelioma	Sarcoma	Total	
Lip	10	—	10	2.7
Tongue	9	—	9	2.5
Larynx	8	—	8	2.2
Esophagus	20	—	20	5.5
Stomach	13	—	13	3 6
Liver	15	—	15	4.1
Gall bladder	10	1	11	3 0
Pancreas	11	—	11	3.0
Colon	13	—	13	3.6
Rectum	11	—	11	3.0
Bladder	80	5	85	23.3
Cervix uteri	12	—	12	3.3
Corpus uteri	9	—	9	2.5
Ovaries	8	—	8	2.2
Breast	15	—	15	4.1
Kidney	5	4	9	2.5
Retroperitoneal	—	27	27	7.4
Mediastinum and lung	2	13	15	4.1
Brain and dura	—	6	6	1.6
Thyroid gland	3	1	4	1.1
Other parts of the body	—	54	54	14.8
Total	254	111	365	100 0

CANCER MORTALITY BY SITE

Tabulations of cancer deaths by anatomical site and by sex are available for the years 1931-1942, inclusive, and are summarized in Table III. The male genital organs comprised the most frequent location of fatal cancer with 1,451 deaths (aside from the catch-all group of "other and unspecified" organs). Deaths from malignant disease of the stomach and duodenum accounted for the next largest number, 1,337. These deaths were approximately evenly divided between males and females. Disease involving the breast and uterus each accounted for somewhat over 1,200 deaths, while cancer of the liver and biliary passages, and of the respiratory tract, follow in order. Over twice as many deaths from cancer of the respiratory tract were found for males than for females, which was true also of cancer of the buccal cavity and pharynx. More deaths from malignant disease in other and unspecified organs were recorded for females than males, possibly because of a less precise diagnosis of cancer in the Mohammedan women of Egypt.

The trend of cancer mortality by site over the period 1931-1942, inclusive, is shown in Fig. 3. Rates for cancer classified as "other organs of the digestive tract" (Table III), of the male genito-urinary system, and of the respiratory system have shown a marked increase since 1936. A somewhat erratic but definite increase in mortality from cancer in other and unspecified organs is also shown. Mortality from malignancy of the esophagus, stomach and rectum, and of the breast shows a slight decrease, but not a significant one. Mortality from cancer of the skin decreased from a level of 0.3 per 100,000 during 1931-1935 to a rate of 0.1 in the years 1936-1942. No trend is evident in the death rates from cancer of the liver and biliary passages, of the uterus, and of the buccal cavity.

Some indication is given in these data of an increase in the mortality of cancer of the deep-seated organs (*i.e.* of the intestines and the male genito-urinary tract) and a decrease or leveling-off of deaths from cancer of the more accessible parts of the body (skin, uterus, and buccal cavity). If these facts can be interpreted in the same manner as similar findings in the United States, they may be cited as evidence of an improvement in diagnosis. The anti-bilharzial hospitals, stationary and mobile, increasing in number during the period, and examining large groups of the population, must have helped to reveal and record cases of cancer of the more deep-seated organs which otherwise would have been easily missed.

The distribution of Egyptian cancer deaths by site is compared with similar distribution for England and Wales and for the United States in Table IV. Although roughly comparable, it is seen that the site most frequently recorded, digestive organs and peritoneum, comprise only 34.4 per cent of the total as against 52.7 and 45.9 per cent in England and Wales, and in the United States, respectively. Deaths comprising the somewhat anomalous grouping, "male genital organs and urinary

TABLE III. CANCER DEATHS AND DEATH RATES BY SITE AND BY SEX FOR THE 12-YEAR PERIOD, 1931-1942. EGYPTIAN REGISTRATION AREA

Site	Deaths			Annual rate/100,000		
	Male	Female	Total	Male	Female	Total
Buccal cavity and pharynx	388	164	552	1.4	0.5	1.0
Digestive tract and peritoneum						
Esophagus	67	29	96	0.2	0.1	0.2
Stomach and duodenum	683	654	1337	2.4	2.3	2.4
Rectum	296	144	440	1.0	0.5	0.8
Liver and biliary passages	847	836	1683	3.0	2.9	2.9
Pancreas	77	40	117	0.3	0.1	0.2
Peritoneum	43	50	93	0.1	0.2	0.2
Other organs, including intestine	525	372	897	1.8	1.3	1.6
Respiratory tract	747	269	1016	2.8	1.0	1.9
Uterus	——	1216	1216	—	4.3	2.1
Other female genital organs	——	98	98	—	0.4	0.2
Breast	48	1181	1229	0.1	4.1	2.1
Male genital organs	1451	——	1451	5.0	—	2.5
Skin	52	46	98	0.2	0.2	0.2
Other nonspecific organs	1078	1205	2283	3.8	4.2	4.0
Total	6302	6304	12606	22.0	21.9	22.1

organs of both sexes," form nearly twice as high a proportion of cancer deaths as in the other two countries. The greater difference, however, is to be found for the catch-all grouping of "other and nonspecified organs," which bulks greatly over the similar figures for the other countries. If this rubric were reduced to comparable size through more accurate reporting and better diagnosis, it is probable that the distribution of cancer deaths by site in Egypt would conform very closely with those for England and Wales, and the United States.

CANCER MORTALITY BY AGE AND SEX

The distribution of cancer deaths by age and sex is available for the registration area of Egypt for the year 1942 (Table V).

Deaths are fairly evenly proportioned between the sexes for each age group although an excess is shown for the males for ages 10 to 24 and 45 to 54, and for the females for ages 55 to 64 and 75 years and over. The death rates by age groups increase over the life span from 1.4 for ages under 10 years to 200.0 for ages 75 years and over. Contrasted with similar rates for the United States and for England and Wales for comparable years, rates for Egypt are the lowest for each age group. The difference, however, is less at the younger ages than at the older ones; at ages under 55 years the rates for Egypt are about half those for the other two countries. Above the age of 55, however, the disparity increases until the rate for 75 years and over is only about one-sixth of the corresponding rate for the other two countries.

TABLE IV: PERCENTAGE DISTRIBUTION OF CANCER DEATHS BY ANATOMICAL SITE, EGYPTIAN REGISTRATION AREA, 1931-1942, ENGLAND AND WALES, 1938-1944, AND THE UNITED STATES, 1943

Anatomical site	Egyptian Regis. Area 1931-1942			England and Wales* 1938-1944			United States† 1943		
	Males	Females	Total	Males	Females	Total	Males	Females	Total
Buccal cavity and pharynx	6.2	2.8	4.5	7.0	1.5	4.1	5.4	1.3	3.2
Digestive organs and peritoneum	40.2	28.0	34.4	57.8	47.9	52.7	52.3	40.1	45.9
Respiratory system	11.8	4.6	8.4	16.0	4.1	9.9	11.3	3.1	7.0
Uterus	——	20.9	10.0	——	12.4	6.4	——	19.4	10.1
Other female genital organs‡	——	1.7	0.8	——	6.5	3.4	——	5.3	2.8
Breast	0.8	20.5	10.2	0.2	19.8	10.3	0.3	18.2	9.7
Male genital organs	23.0	——	12.0	7.5	——	3.7	12.3	——	5.8
Urinary organs				4.7	2.4	3.5	6.2	3.2	4.8
Skin (scrotum excepted)	0.8	0.8	0.8	1.7	1.3	1.5	2.5	1.5	2.0
Brain and other parts of nervous system				1.3	0.9	1.0	2.0	1.1	1.5
	17.2	20.7	18.9						
Other or unspecified organs				3.8	3.2	3.5	7.7	6.8	7.2
Total, all sites	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* These data were computed from data kindly supplied to me by the Rt. Hon Lord Amulree, Ministry of Health, Whitehall, London.
† These data were computed from data kindly supplied to me by Mr. J. C Capt, Director of the Bureau of the Census, Department of Commerce, Washington, D. C.
‡ The data, for Egypt, include female urinary organs.

TABLE V: DEATHS IN THE EGYPTIAN REGISTRATION AREA BY AGE AND SEX, 1942; WITH AGE SPECIFIC DEATH RATES COMPARED WITH THOSE OF THE UNITED STATES, 1940, AND ENGLAND AND WALES, 1942

| Age | Deaths, Egypt Regis. Area, 1942 | | Total | | United States 1940 Rate* | England and Wales 1942 Rate* |
	Male	Female	Number	Rate*		
0– 9	10	8	18	1.4	3.8	3.5
10–24	29	16	45	3.3	4.6	4.5
25–34	34	39	73	10.2	17.3	18.1
35–44	95	93	188	31.1	61.1	63.6
45–54	154	137	291	76.2	168.8	186.1
55–64	156	174	330	142.2	369.6	419.9
65–74	113	103	216	171.4	695.2	824.7
75 and over	68	86	154	200.0	1183.9	1162.7
All ages	659	656	1315	27.2	120.3	183.4

* Per 100,000 population

NOTE: The rates for this table were computed by the use of population estimates for each group secured by apportioning the estimated population for the registration area according to the age distribution of the 1937 census of Egypt. Accuracy of the estimates depends upon the agreement between the age distribution of Egypt and that of the registration area for the country. Data necessary for this comparison are not available.

It was previously pointed out that the age distribution of the population of Egypt differs from that of the United States or of England and Wales in having a greater percentage of younger persons and a smaller percentage of older persons. The difference thus operates in the direction of a smaller total cancer death rate for all ages. The extent of the age effect can be shown. If the age distribution of Egypt had been the same as that for the United States in 1940, and the cancer death rates for Egypt shown in Table V were operative, the mortality rate from cancer for all ages would have been 39.8 instead of 27.2. The increase of 12.6, or 46 per cent, although large, clearly shows that the factor of age does not account for the relatively small total cancer death rate recorded for Egypt.

CANCER MORTALITY COMPARED WITH THAT OF OTHER DISEASES

In view of the low cancer mortality rate in Egypt compared with that of other countries it is interesting to note that an entirely different situation exists with regard to mortality from all causes of death. We have available for study the total recorded deaths in the registration area of Egypt for the period 1918-1942 and a tabulation by cause of death for the ten year period 1932-1941. The average annual mortality rate for all causes was 32.2 per 1,000 population for the period 1918-1942; for the period 1932-1941 in the expanding registration area, the rate was 36.8. Similar rates for the United States and for England and Wales in 1940 were 10.8 and 13.8 respectively (exclusive of deaths of civilians due to operations of war in England and Wales). The death rate for all causes in Egypt is thus approximately three times as great as that for the United States, and for England and Wales, in spite of a presumed under-registration of deaths and a very favorable age distribution.

Death rates for the principal causes of death are shown in Table VI. Diarrhea and enteritis, the respiratory diseases, tuberculosis, the other infections and parasitic diseases, heart disease, and the nonvenereal urinary and genital diseases all rank higher in the list than cancer. The *prima facie* evidence of this table points to a low prevalence of cancer in Egypt.

However, other facts lead us to accept this evidence with great caution. Cancer outranks nearly all the other diseases on the list in difficulty of diagnosis. The excessive rates for the rubrics "senility" and "other causes" indicate that, in general, diagnostic certification of deaths is poor in Egypt. These two groups together form 28 per cent of all deaths in Egypt in contrast to 13 and 10 per cent in the United States and in England and Wales, respectively. It is a well-known fact that in regions where medical service and death certification are poor, assignments of cause of death to senility and to "ill-defined causes" (included in Table VI in the "other cause" rubric), are high. It will also be recalled from the preceeding paragraphs, that criticism of death registration in Egypt is in general directed towards certification of cause of death, and not towards the actual enumeration of numbers of deaths.

Further, it is easy to establish the fact that the rate of cancer mortality is markedly low in areas of defective death registration. As an illustration, tables of mortality were examined for 52 counties in 10 states in the United States in which registration was notably poor in 1920. The cancer death rate per 100,000 was 12.5 in this group of counties, half that of the rate in Egypt (23.3) in the same year, and but 15 per cent of the cancer death rate in the American death registration area in 1920. The point seems clear that a low level of cancer mortality can be accepted only after abundant demonstration that the mechanism of death certification and reporting is well set up and functioning properly.

It is interesting, before closing the present section, to compare the various causes of mortality in Egypt with those of the United States, and of England and Wales, as shown in Table VI. The outstanding contrast is for diarrhea and enteritis; mortality from this cause is 10 times greater in Egypt than in the other two countries. Excessive mortality is also indicated for the respiratory

TABLE VI. DEATH RATES FROM PRINCIPAL CAUSES OF DEATH IN THE EGYPTIAN REGISTRATION
AREA 1932-1941, THE UNITED STATES, 1940, AND IN ENGLAND AND WALES, 1940

Cause of death	Annual rates, 100,000 population		
	Egypt 1932-1941	United States 1940	England and Wales 1940
Tuberculosis	66.1	45.9	69.9
Syphilis	10.2	14.4	8.0
Malaria	1.3	1.1	0.1
Dysentery	10.9	1.9	0.5
Other infections and parasitic diseases	124.9	27.7	52.0
Cancer and other malignant tumors	26.9	120.3	172.3
Nonmalignant or unspecified tumors	1.7	5.0	5.7
Diabetes mellitus	19.4	26.6	13.4
Pneumonia	307.9	54.9	72.7
Bronchitis	258.6	3.0	115.9
Other respiratory diseases	64.5	8.2	21.5
Cerebral hemorrhage	24.5	88.2	124.2
Hemiplegia and other paralyses of unstated origin	38.3	2.7	5.2
Heart disease	124.3	292.5	341.6
Other circulatory disease	17.0	21.9	39.4
Disease of the liver and biliary passages	17.8	15.8	6.0
Urinary and genital diseases, not venereal	135.0	95.4	60.4
Puerperal mortality	16.1	6.7	4.1
Diarrhea and enteritis	1255.2	10.3	11.1
Senility	347.7	7.7	48.2
Violence	124.4	94.3	118.3
Other causes	683.3	131.9	89.4*
All causes	3675.7	1076.4	1379.9*

* Exclusive of deaths of civilians due to operations of war.

diseases although this finding is hardly established by the data of Table VI, since the rates for the other two countries are only for 1 year. However, during the 9 year period 1932-1940 the rates for respiratory disease varied between 66 and 105 in the United States, and between 145 and 210 in England and Wales so that the respiratory disease rate of 631 for Egypt is approximately 6 times that for the United States and 3 times that for England and Wales.

Deaths from chronic disease are low in Egypt. The rates for cerebral hemorrhage, and circulatory disease, are lower in Egypt than in the other two countries, while that for the non venereal urinary and genital diseases is somewhat higher. This group of diseases together, and including diabetes, shows a mortality rate of 320 per 100,000 in Egypt as against 525 and 579 for the United States and for England and Wales, respectively, for the years indicated.

NATIONALITY AND RELIGION

The foreign born population in 1937 included 187,000 persons or 1.2 per cent of the total population, concentrated in the urban centers.

Table VII gives the distribution of deaths from cancer and from all causes by nationality for the 25 year period of 1918-1942, together with the cancer death rate per 100,000 for each nationality of

foreign-born. The rates show a wide variation from 14.5 for native Egyptians to 64.8 for the Italian foreign-born. The rates for the foreign-born, with the exception of the "other foreign-born" group, are all more than twice as great as for native Egyptians.

The mortality rates for all causes, on the other hand, are high (33.8 per 1,000) for native Egyptians and low (ranging between 9.1 and 12.4 exclusive of the "other" group) for the foreign-born. The rates for the foreign-born are much as one would expect in an average European population.

The variation in cancer mortality according to nationality is in part due to differences in the age distribution. Thirty-one per cent of the foreign-born population were listed as under 20 years of age in the census of 1937, contrasted with 48 per cent for native Egyptians, whereas the percentage of the population over 50 years of age was 18 for the foreign-born as against 12 for natives. The differences in age are large, but cannot account for the higher incidence of cancer mortality among the foreign-born.

The foreign-born populations reside in the urban areas; 80.0 per cent of the total foreign population was recorded as resident in Alexandria and Cairo in 1937. A classification of the census returns by occupation shows that 39 per cent of all occupied foreign-born were employed in industry and com-

TABLE VII: CANCER MORTALITY IN THE EGYPTIAN REGISTRATION AREA, CLASSIFIED ACCORDING TO NATIONALITY; 25-YEAR PERIOD, 1918-1942

| | Egyptians | Foreign-born | | | | | | Total Population |
		Total	British	French	Italian	Greek	Others	
Cancer deaths in 25 years	17,135	2,761	417	236	843	1,046	219	19,896
Deaths from all causes in 25 years	2,819,026	51,247	10,565	4,981	13,755	17,806	4,111	2,870,303
Cancer deaths per 1,000 total deaths	6	54	39	47	61	58	53	7
Population, Egyptian Regis. Area, in mid-period	3,336,400	225,006	34,169	24,332	52,462	76,264	38,373	3,562,000
Cancer death rate/100,000	14.5	48.8	49.7	38.8	64.8	55.1	23.4	22.3
Death rate from all causes/1,000	33.8	9.1	12.4	8.2	10.5	9.3	4.3	32.2

merce, as against 12 per cent of the occupied Egyptian population. Of the latter, 58 per cent were engaged in agriculture and fishing, as against 1.3 per cent of the foreign-born. A marked contrast thus exists between the native Egyptians and the foreign-born population; the former are rural dwellers, the latter are urban. Any comparison of mortality rates between the two groups must be made in consideration of the manner of death certification. It has been demonstrated previously that mortality reporting in Egypt is most complete in urban areas and is defective in rural ones. This fact alone is enough to account for the differences shown in Table VII.

Nationality differences in mortality in Egypt must be accepted with great caution until it is abundantly demonstrated that the system of death reporting and certification is complete.

The 19,896 deaths recorded during the period 1918-1942 are reclassified according to religion in Table VIII. Mortality rates for cancer are lowest among the Moslems, 16.3 per 100,000; next highest among the Christians, 80.5; and highest among the Jews, 187.4. In contradistinction to the rates for the foreign-born, the all-cause mortality rates vary in the same manner, though to a lesser degree.

Information is not available to give an indication of the urban-rural or the occupational distribution of the population by religion. It would appear evident, however, from a comparison of Tables VII and VIII that the Christian and Jewish populations of the latter table must include a number of areas in which mortality is exceedingly high. The foreign-born populations shown in Table VII are largely Christian or Jewish; their annual death rate is 9.1 per 1,000. Table VIII shows that the Christian and Jewish rates for all causes are 40.5 and 59.9, respectively! Further investigation must be carried out in order to clarify the results shown in Table VIII.

OCCUPATIONAL CANCER

It may be conjectured that the greater exposure of the foreign-born to the irritants and injuries of industry renders them more liable than Egyptians to attack by cancer. However, the number of foreign-born working in certain industries in which exposure to carcinogenic substances is suspected is too small to account for the results shown in Table VII. The number of such industries is quite limited in Egypt and it is only rarely, if at all, that the so-called occupational cancers are encountered in Egyptian medical practice, among either Egyptians or foreign-born.

Chimney sweep's cancer was never seen in Egypt; it has even ceased to occur in England and Wales, where it was first noticed, after the introduction of modern methods of cleaning chimneys. Cancer developing after long contact with coal and wood tars, occasionally noted in Europe and America, is unknown in Egypt. Persons trading in coal and coke are largely Egyptians and do not exceed 5,000 in number, while the number of persons preparing pitch for the maintenance of roads is also small. Malignancy developing on benign epidermal lesions of the scrotum, forearms and legs of persons engaged in paraffin works is also unknown in Egypt. Cancer in brickmakers, developing as epitheliomas in old skin lesions, is not known in Egypt, although about 7,000 persons are engaged in this occupation.

Cancer in cotton spinners, chiefly attacking workmen who run spinning machines and in the process become covered with mineral oil, is also absent in Egypt. In a cotton spinning factory in Alexandria, employing between 5,000 and 10,000 workmen under strict medical supervision, not a single case of cancer of this type was observed over a period of 12 years. The number of workmen in cotton spinning and weaving in Egypt was 28,000 in 1937; nevertheless no cases of cotton spinners' cancer have been recorded.

Miners, who do not exceed 800, petroleum refiners numbering about 1,500, and aniline dyers numbering about 5,000, also appear to be free from the cancers associated with these trades. The type of cancer found among aniline dyers is of interest to Egyptian cancerologists because of its re-

TABLE VIII: CANCER MORTALITY IN THE EGYPTIAN REGISTRATION AREA, CLASSIFIED ACCORDING TO RELIGION; 25 YEAR PERIOD, 1918-1942

	Moslems	Christians	Jews	Others	Total
Cancer deaths in 25 years	13,189	5,941	747	19	19,896
Deaths from all causes in 25 years	2,544,338	299,428	23,976	457	2,870,303
Cancer deaths/1,000 total deaths	5.2	19.8	31.2	41.6	6.9
Population, Egypt. Regis. Area in mid-period	3,244,982	295,646	16,009	5,343	3,562,000
Cancer death rate per 100,000	16.3	80.5	187.4	14.2	22.3
Death rate from all causes per 1,000	31.4	40.5	59.9	3.4	32.2

semblance to cancer of the bladder developing in connection with bilharziasis, one of the most frequently occurring types of cancer in Egypt.

X-ray cancer is also unknown in Egypt. I have not seen or heard of a single case in 20 years of practice in Egyptian hospitals, either among radiographers and physicians or among patients treated by radiotherapy, although in the provinces, x-rays are often handled by inexperienced persons.

BILHARZIASIS AND CANCER

Bilharziasis attacks from 30 to 90 per cent of the population, according to locality, and is most prevalent in rural areas, small towns and villages.

In the series of 5,924 autopsies in Kasr-el-Ainy Hospital, Cairo, 1905-1930, analyzed by Professor Sorour (16), malignant disease was found in 20.6 per cent of the cases of bilharzial bladder and in 4.4 per cent of cases of bilharzial cirrhosis of the liver. In the same series, bilharziasis and cancer were associated in 16.6 per cent of the cases of cancer of the colon. The malignant bladder cases, 85 in number, were all associated with bilharziasis.

Dr. M. Ashour (7) and Dr. Anis Onsy Bey, examining a series of pathological specimens in the Ministry of Public Health Laboratories, found in 3,180 specimens of malignant disease 117 or 3.6 per cent showing bilharzial pathological changes. The bilharzial malignancies were 8 malignant papillomas, 104 carcinomas (46 of which were vesical, or 45 per cent) and 5 sarcomas.

These and similar data have been taken by various authors as proof of a definite relationship between bilharziasis and the development of cancer in certain parts of the body, especially the bladder, prostate and colon where malignant disease is often found to develop on old-standing bilharzial lesions. Such findings are termed bilharzial cancer. The parasitic infection is not credited with provoking malignant disease, but the irritation of the parasites and their ova and embryos, with the coincident chronic sepsis is suspected of being responsible.

Dolbey and Mooro (9) studied 671 cases of cancer in various sites and found malignant disease in a large number of bilharzial bladders but none

in bilharzial renal pelves, ureters and intestinal tracts. They noticed that the large intestine was often the seat of large benign growths, resulting from chronic irritation induced by the parasites, which rarely developed malignancy. They ascribed this to the acid reaction of the bowels and absence of chronic sepsis such as that found in cystitis, and suggested that the high incidence of malignancy in bilharzial bladders was due to irritation of the ova in an alkaline medium such as the stagnant urine of bilharzial cystitis. Onsy (13), Sorour (17) and Barsoum (8), however, in biopsies and autopsies found an appreciable number of cases of cancer not only in bilharzial bladders but also in bilharzial prostates, testicles, small intestine, colons, rectums, livers, uteri, ovaries, vulvas, vaginas, etc. Any explanation, therefore, of the role of bilharziasis in the development of malignant disease must not be limited to the bladder, but must include other sites as well. This fact has led to acceptance only that chronic irritative action of the bilharzial ova and embryos and of the long-
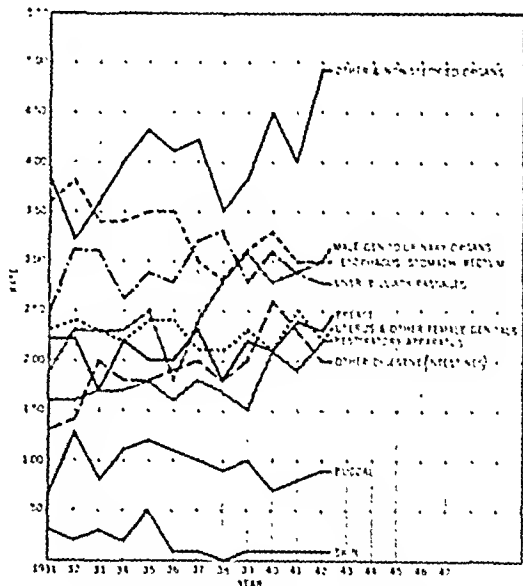

FIG. 3.—Cancer death rate per 100,000 of population in Egyptian Registration Area by organs in 12 years 1931-42.

standing inflammatory processes resulting from the parasitic infections are precursors of malignancy in bilharzial cancer.

This concept is strongly held by the majority of writers on cancer in Egypt. However, evidence of an association between bilharziasis and cancer would be difficult to avoid in a country so heavily infected with bilharziasis. Proponents of the theory of the causal relation of the disease to cancer have yet to show that the incidence of cancer is higher in a group of definite bilharzial cases than in one in which this disease is definitely absent but is similar to the first in the other pertinent factors of age, sex, race, occupation, etc. Further, unless a definite carcinogenic agent, or other predisposing factor, is proven to exist in bilharziasis, the term "bilharzial cancer" is unjustified. Investigations of this kind have not been carried out, to my knowledge, in Egypt, and the field is still quite open for research.

SUMMARY

Compared with rates for other countries, the recorded incidence of cancer mortality is low in Egypt, but death registration in the country is such that the low rate for Egypt can hardly be accepted. Before this can be done it must be abundantly demonstrated not only that all deaths within the designated registration area are reported, and the cause of death certified, but also that the certification is made as often as possible by a qualified physician with medical knowledge of the case before death. This can be accomplished in part by intensive and concerted action by the medical profession and the health authorities to secure a better registration system. Further improvement than this, however, must await a more complete development of medical care in Egypt, not only with respect to an improvement in the qualifications of the profession, but in the acceptance by the people of the medical profession as the only group qualified to diagnose and treat the ill.

It is likely that most of the differences noted between specific rates for sex, race, and areas are also due to defective reporting and certification.

Until medical practice and death registration are improved, it would appear that evidence of the actual amount of cancer in the country can best be secured by a series of well planned and conducted surveys by qualified medical investigators working in conjunction with experts in the field of statistical sampling. The promise of such surveys is great. The many contrasts of the social, occupational,

racial, religious and dietary aspects of life in Egypt offer opportunity for comparisons which would yield valuable information concerning cancer.

The relation between bilharziasis and cancer is still uncertain and needs thorough and careful study, both from the point of view of the incidence of cancer in bilharzial cases and of the pathology and chemistry of the two diseases.

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Book Reviews

CANCER AND OCCUPATION IN DENMARK. 1935-1939. Johannes Clemmesen. Translated by Robert Fraser. Copenhagen: Nyt Nordisk Forlag—Arnold Busck. 1941. 75 pages.

In this interesting analysis of deaths from cancer in their relation to occupation occurring in Denmark during 1935 to 1939, Clemmesen points out that cancer research which was dominated during the past century by pathologists has been moved into the laboratory and has become to a large extent the domian of chemists, radiobiologists, virologists and geneticists. The important direct contact with the practicing physician has been lost thereby. Clemmesen believes this to be unfortunate since a part of the future fight against cancer will be prophylactic, and research for this reason should deal with the etiology of cancer in man. While statistical mass observations on this point are of limited value, they may provide clues to other profitable approaches to the cancer problem.

Denmark which has the highest annual mortality from cancer of all countries (14 cancer deaths per 10,000 inhabitants) offers certain advantages for statistical observations on the relation between cancer and occupation. These are, high longevity of the population permitting the unsually long latency period of cancer to take effect; a well organized and evenly distributed medical service and high medical standards; accessibility of all social classes to the same high grade specialized medical care in three modern radium centers; good communications throughout the country; homogeneity in geographic, climatic and ethnologic respects, small social and economical differences; stable and uniform occupational conditions in different parts of the country over many years; a comparatively moderate number of cases allowing a detailed analysis; death certificates filled out by physicians, based to a high proportion (about 50 per cent) on elaborate hospital and laboratory data, and representing legal documents.

Cancer mortality standardized for age groups was found to be higher in towns than in rural districts and higher in men than in women. The analysis was restricted to the male population, as great technical difficulties exist in this respect for applying such an analysis to gainfully employed women and married women. For the purpose of the analysis the cancer cases according to total number and according to organ affected were distributed among the 7 occupational groups used in the census taken in 1930 in Denmark. There was a low mortality from all forms of cancer in agriculture and a high cancer incidence in industry in persons belonging to the age group 45 to 64 years. However, the respective mortality rates from cancer in the two occupational groups ran parallel to their mortality rates from other causes. Although deaths from cancer at ages over 65 years constituted the same fraction of the total mortality in the different occupational groups, the local distribution of cancers varied in different groups. This observation suggested that in Denmark also occupation had a definite

bearing on the development of cancers. While it was not possible to determine with certainty from the material available if there was a corresponding, even distribution of the cancer mortality between the different occupational groups in the age group of 45 to 64 years, it is not improbable that the latency period for the development of tumors is different in different occupations depending upon the carcinogenic potency of the injurious agent. Predisposed individuals having a tendency toward rapid cancer development were probably equally distributed among the different occupational groups. The question of the inter-relation between exogenous factors and hereditary disposition to cancer is considered of greatest importance for the possible prevention of malignant tumors.

Cancer of the stomach which accounted for 31.9 per cent of the total cancer mortality occurred with almost average frequency in the agricultural group age 45 to 64 years, while it was found at an excess of 129 per cent in the industrial group of the same age. Gastric cancer mortality was low in all age groups of the group comprising the professional and white collar class, where it stood at 57.1 per cent of the average in the age group 45 to 64. These differences were attributed to better general hygienic conditions in this class and not to the effect of some carcinogenic factors acting on the stomach of the members of the industrial group. These observations confirmed similar ones previously made in England on the uneven distribution of gastric cancer on five social groups. In England it was proposed therefore to reduce gastric cancer mortality by improving the living conditions of groups IV and V with lowest economical standards to those prevailing in groups I and II which have a low gastric cancer mortality. Unfortunately, well-to-do people were found to have a higher intestinal cancer rate than persons belonging to the socially and economically low classes. Clemmesen suggested that the differences in the local distribution of cancers of the gastrointestinal tract might be causally related to the higher amounts of foodstuffs ingested by the physically laboring classes, as there is no material differences in the quality of food consumed by all occupational groups in Denmark. Esophageal cancer incidence was high in the industrial group and low in the agricultural and white collar groups. However, by far the highest incidence of esophageal cancer was noted in persons belonging to the various alcoholic trades (waiters, restaurant and hotel owners and managers, commercial travellers, packers) confirming again in this respect previous English observations. Rectal cancers, on the other hand, were infrequent in members of the alcoholic trades, but high in warehouse men and packers. Both intestinal and pulmonary cancers were excessively frequent in the industrial group and occurred below the average figure in the agricultural group. Clemmesen stated that the tar theory of lung cancer based on the construction of tarred roads in recent years is premature, as not sufficient time

has elapsed since the introduction of such roads to have exerted any definite influence on the incidence of lung cancer.

As the discrepancy between cancer morbidity and cancer mortality rates is apt to grow with the increasing improvement of therapeutic measures, Clemmesen proposed to make cancer a notifiable disease. The information should contain exact identification of the patient and of the cancer as to site and type, histological diagnosis, occupation, and cause and place of death. Information as to the cause of death in cured cases is regarded by him as especially important. The public should be taught to avoid intimate contact not only with bacteriological dirt but also with chemical dirt, such as grease and lubricating oils. The conduct of similar surveys in other countries Clemmesen considered as valuable and indicated.

It is obvious that surveys of this nature are important and urgently needed in this country because of its leading position as an industrialized country. Information on the relation between cancer and occupation is moreover here highly defective, although the practical and scientific significance of exogenous and especially occupational agents in the genesis of cancer has become increasingly evident in recent years. There can be no doubt that a comprehensive study of the workers exposed to occupational carcinogenic agents, and affected by industrial cancers, would be of eminent value for future control not only of this type of cancer but of cancer in general.

W. C. HUEPER

THE CHEMICAL KINETICS OF THE BACTERIAL CELL. C. N. Hinshelwood. Oxford: Clarendon Press. 1946, x + 284 pages. Price $6.75.

The author of this book is a recognized authority on the theoretical aspects of chemical kinetics, particularly those of gaseous reactions. In recent years he and his students have become interested in the formal analogy presented by the kinetics of bacterial growth. This volume is avowedly an attempt to ascertain whether the laws governing known chemical reactions can be used to describe quantitatively the normal growth of bacterial cells and their adaptation to various environmental influences.

From the biological viewpoint the presentation would be clearer had it begun with Chapter VII (adaptation) and continued with the remaining sections on variation, selection and cell division, ending with Chapters I to VI in which the author's views on kinetics and adaptation are developed. According to the latter, 'the adaptive response depends on a shift in the enzyme balance of the cell. Although mentioned in the later chapters, the alternative theories of mutation and selection receive rather cursory and inconclusive treatment. It is not our intention to enter into the merits of these rival theories here; nevertheless, it should be pointed out that important objections to the author's theory presented by recent experiments of Demerec and of Luria on penicillin and sulfonamide resistance receive no comment in spite of the fact that Demerec's paper is cited in the bibliography as a typical example of drug resistance.

The value of the book lies in its coordinated presentation of the author's own excellent quantitative data on adaptation, and the extended mathematical development of a kinetic theory derived from the concept of a shift in enzyme balance. Although in its present form the theory is not concerned with phenomena other than the growth of bacterial cells there appears to be no reason why an analogous theory could not be developed to account for certain aspects of normal and abnormal tissue cell growth. The main difficulty would appear to be in establishing whether the basic assumptions which the author postulates for his theory are really valid for any given application. Even for bacterial systems there is evidence that the mere correspondence between predicted and experimental values is not a crucial test for the validity of the theory.

HENRY P. TREFFERS

Experimental Studies on the Pathogenesis and Histogenesis of Ovarian Tumors in Mice*

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The role of the anterior pituitary in the regulation of the gonadal function, and the reciprocal influence of the gonads on the pituitary gland have been established (81, 93). Gonadotrophic and ovarian hormones, under normal circumstances, maintain a state of antagonistic interaction (60). It is not difficult to understand, however, that certain conditions may arise that disturb the usual secretory activity of one or more of these endocrine glands thus resulting in hormonal imbalances. The prolonged exposure of animals of some species to estrogens results in the appearance of atypical growths or cancers in some genital tissues and in other organs (31). The nature of the neoplastic action of estrogens, whether estrogens act directly on the body tissues or indirectly through the disturbance of the production of pituitary hormones or otherwise, still awaits the results of further study.

The influence of pituitary hormones on the development of tumors has been studied by the use of animals given injections of different pituitary preparations, and by observing the effect of hypophysectomy on tumor-bearing animals or animals given carcinogenic substances. These investigations so far give little conclusive information (79). Recently, Pfeiffer and Hooker (69) obtained small local overgrowths of testicular interstitial cells in mice of the A strain that were given daily injections of pregnant mare's serum for several months. These growths resembled early stages in the development of the interstitial cell tumors that have been induced in estrogen-treated mice of this strain. The difficulty of obtaining purified and

standardized gonadotrophic hormones, and the formation of antihormones have impeded adequate study of the long-term effects of gonadotrophins.

The present experiments are based on two principles: (a) the capability of the liver to inactivate ovarian hormones, i.e. estrogen and progesterone, when the hormones circulate through the hepatic portal system (6, 17, 35, 45, 46, 55, 77, 78, 91); and (b) the increase of intrinsic gonadotrophic hormones subsequent to castration as determined by bioassay of urinary, blood and hypophyseal gonadotrophins, and in experimental parabiosis of an intact with a castrated or roentgen-rayed animal (20, 21, 23, 28, 54). A condition of endocrine imbalance might be produced by the transplantation of ovaries into spleens of castrated mice. Such conditions should permit the study of continuous actions of endogenous pituitary gonadotrophins in ovarian tumorigenesis. Biskind and Biskind (4) reported that tumors developed in ovaries transplanted into spleens of 3 castrated female rats. Lipschütz and associates (52) observed only growth of lutein cells in some female guinea pigs with intrahepatic and intrasplenic grafts of ovaries.

Following the exposure of immature rats to roentgen irradiation (459 r or 574 r) directed to abdominal fields overlying the ovaries, Drips and Ford (18) observed hyperplasia and ingrowth of the germinal epithelium as well as the development of atypical, hyperplastic structures of epithelial cells that assumed an acinar arrangement and resembled "carcinomatous ovarian cystadenomata of the ovary of human beings." They found that such hyperplastic follicular structures occurred 4 months after irradiation, but more extensive growth had taken place in the rats that were killed 8 months later. The formation of granulosa-cell tumors, luteomas, and "tubular

adenomas" in mice subsequent to similar applications of roentgen rays has been reported by Furth (29) and others (34, 85), and the histogenesis of the ovarian tumors has also been studied by several workers.

The present investigation is a study of the pathogenesis and the possible histogenesis of the ovarian tumors appearing in castrated mice bearing the intrasplenic ovarian transplants. A preliminary note on these tumors has been reported elsewhere (50).

MATERIALS AND METHODS

Inbred mice of the Strong A and C3H strains,[1] and hybrid mice (AC3, AC5)[2] were used. They were kept in an air-conditioned room and fed a commercial diet (Nurishmix) and water with occasional supplements of grain (a mixture of wheat, oats, and sunflower seeds) and calf-meal pellets. Six groups of experiments were set up (Table I): (a) 21 castrated male mice with homotransplants of an ovary into the spleen; (b) 52 castrated female mice with autotransplants of an ovary into the spleen; (c) 25 castrated female mice with subcutaneous autotransplants of an ovary into the right axillary region; (d) 12 intact male mice with subcutaneous homotransplants of an ovary into the right axillary region; (e) 9 castrated male mice with subcutaneous homotransplants of an ovary either into the right axillary region or into the abdominal region; and (f) 34 intact male mice with homo- or heterotransplants of an ovary into the right testis. Castration and grafting were done in one stage operations. Upon the removal of gonads the Fallopian tubes or sperm ducts and adipose tissues adjacent to the gonads were cauterized. The ovary was implanted into the spleen through a small incision made in the splenic capsule. The method of intratesticular transplantation of ovaries has been described (30). The ages of mice at the time of grafting varied from 1 to 3 months, except in the group (f) in which a few mice were operated upon at the age of 130 days. The donors of the ovaries for homotransplants or heterotransplants were usually younger than the recipients. The experimental animals received no treatment other than as mentioned.

Vaginal smears of the castrated female mice were taken at intervals during the experiment. Laparotomies were performed in some animals to check for adhesions and to determine growth of the graft. At autopsy the ovarian grafts, genital tissues, adrenal glands, kidneys, submaxillary glands, and pituitary glands were examined and preserved for histological examination. Mam-
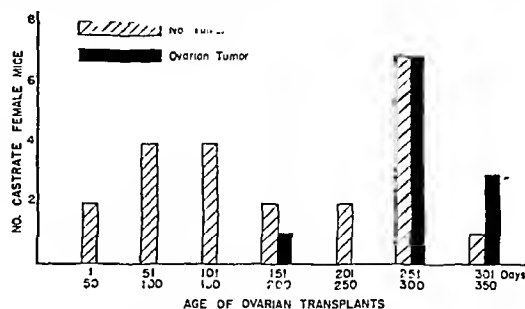

Fig. 1.—Age distribution of ovarian tumors in intrasplenic ovarian transplants in castrated male mice.

mary glands of a few mice were studied as whole mounts. Most of the ovarian grafts were sectioned serially and several areas from the ribbons were saved and stained with hematoxylin and triosin.
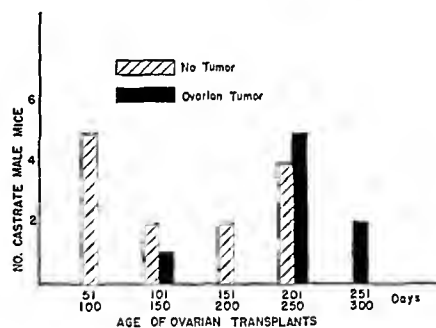
Fig. 2.—Age distribution of ovarian tumors in intrasplenic ovarian transplants in castrated female mice.

OBSERVATIONS

I. Tumors in Intrasplenic Ovarian Transplants

The ovarian transplants grew progressively in the spleens of castrated male and female mice and some were palpable through the abdominal wall at 3 months subsequent to grafting. At this time the transplants, observed at laparotomy, contained many large follicular cysts and they usually measured about 2 × 4 × 5 mm.

The earliest granulosa-cell tumor was discovered in a castrated male mouse bearing a 130 day old graft although most of the tumors occurred in grafts over 200 days old. Most ovarian tumors were larger than the non-tumorous grafts and protruded from the surface of the spleen; the largest measured 5 × 8 × 9 mm. in diameter. The tu-

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[1] These mice were supplied by Drs. A. Gorbman, C. W. Hooker and L. C. Strong.

[2] AC3 are first generation hybrid mice of the following parentage: C3H (low-tumor) ♀ × A ♂.

AC5 are first generation hybrid mice of the following parentage: A (low-tumor) ♀ × C3H ♂.

mors were irregular in shape, reddish yellow, and some showed a few scattered hemorrhagic cysts as indicated by dark red spots (Fig. 4).

A. Castrated male mice bearing intrasplenic ovarian transplants.—Five granulosa-cell tumors, two pretumorous lesions of the same type and one mixed tumor— both granulosa and luteoma cells— were found among the 21 castrated male mice of the A strain (Table I). With one exception these tumorous grafts were more than 200 days of age.
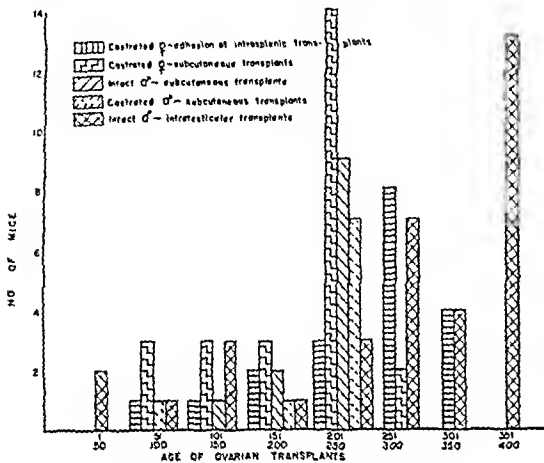

Fig. 3.—Age distribution of ovarian transplants in mice.

Among the 4 castrated male mice that survived over 200 days after the transplantation with no tumor growth only 1 showed viable ovarian tissue, a few primary follicles and some ovarian stroma constituting the small graft. The grafts in 2 other mice were composed of uterine tubal tissue or adipose tissue and in 1 animal no graft was found.

Nine mice that had no tumors of the transplanted ovaries were killed or died when the grafts were less than 200 days old. Two mice that had carried intrasplenic grafts for 170 and 198 days respectively had large transplants showing some ingrowth of the germinal epithelium and a disorganized stroma. The impression was given that, had the hosts survived for a longer period, the transplants would have become tumorous. The transplants in the two mice that had survived from 101 to 150 days were small, one could not be adequately examined because the animal had died sometime before autopsy and the other contained several large follicles and cysts of uterine tubal epithelium. Viable ovarian tissue was found in the 5 mice that had intrasplenic grafts for less than 100 days. The grafts differed in size but all contained follicles, either primary or both primary and secondary. The bulk of the grafts, however, consisted of enlarged interstitial cells with a pale

staining cytoplasm. In only 1 of the later transplants were ingrowths of the germinal epithelium noted (Fig. 1).

Microscopically, the granulosa-cell tumors consisted of cells with hyperchromatic vesicular nuclei. Most of the tumor cells were indistinguishable from the granulosa cells of normal graafian follicle; mitoses were frequent. In some areas the tumor cells invaded the splenic tissue although no metastases were observed. The tumor cells usually formed many somewhat dissociated masses and the arrangement of the cells in these masses varied considerably even in a single section of the same tumor. In some masses the tumor cells were diffuse and in others intermingled with thecal and interstitial cells; but most of the tumors contained extensive one-cell-layered cords that were more deeply stained than the adjacent tumor cells, and that were sometimes arranged in tubular structures (Fig. 5). Enlargement of the tubular spaces appeared to give rise to small and medium-sized cavities that usually contained a few degenerating cells and sometimes blood cells or coagulated fluid (Fig. 6). Also, folliculoid structure seemed to form by separation of a mass of tumor cells into smaller groups each surrounded by delicate connective tissue septa; the cavities resulted apparently by subsequent liquefaction of some of the centrally placed cells (Fig. 5, 7). A layer of spindle-shaped cells, suggesting thecal cells, was observed outside the ovarian follicle-like structures. Accumulation of the tumor cells and false ovocytes occasionally simulated the appearance of a cumulus oophorus (Fig. 7). Thus the granulosa-cell tumors were preponderently diffuse and folliculoid in pattern as featured by Barzilai (3) in human material. Different histological arrangements of the tumor cells co-existed in the same tumor. However, two tumors referred to as the 'pretumorous' lesions were composed of small groups of diffuse granulosa cells and a few hemorrhagic cavities.

A large mass of lutein-like cells (luteoma) was observed in one of the granulosa-cell tumors, as well as many scattered lutein and interstitial cells. Some granulosa-tumor cells seemed to show transformation into lutein cells (Fig. 9). A description of luteomas will be given when the tumors in the castrated female group are described.

The proliferation and ingrowth of the germinal epithelium were observed in all these tumors and in some of the non-tumorous grafts. Local proliferation of the germinal epithelium with evidence of mitotic activity occurred frequently in the tumors, and cells with ellipsoidal nuclei simulating the cells of the germinal epithelium were often mixed with the adjacent tumor cells (Fig. 10).
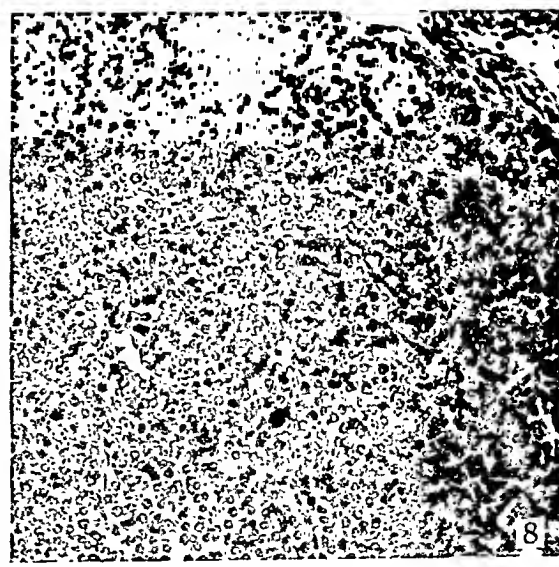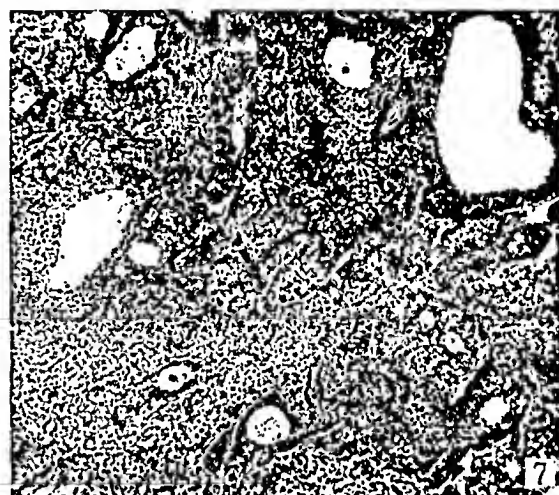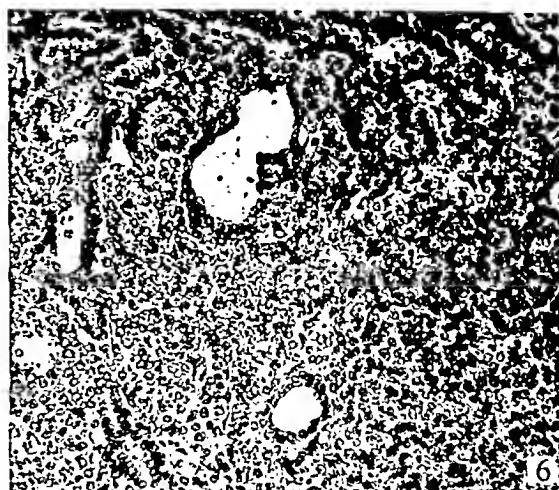
Figs. 4-8

The ingrowth of germinal epithelium formed groups of networks of tubules or cysts that sometimes contained blood cells as seen in the folliculoid structures. Many cells identified as granulosal, lutein, or interstitial cells, and primary ovocytes were observed among the network of epithelial ingrowths (Fig. 11). In some grafts the nuclei of the epithelial cells that lined the tubules appeared to become vesicular and the whole mass of epithelial ingrowth resembled the folliculoid formations (Fig. 12). An intimate connection between the epithelial ingrowths and cells of the tumors was discerned (Fig. 13).

The seminal vesicles and prostates were atrophied in the castrated male mice bearing intrasplenic ovarian grafts, and histological examination of the seminal vesicles revealed no indication of hormonal stimulation. The adrenal glands showed degeneration of the x-zone. No detectable hormonal effect on the kidneys was noted and the parietal layer of the Bowman's capsule was usually composed of squamous epithelial cells. The terminal tubules of the submaxillary glands were lined by low epithelial cells, but the percentage of total tubular and alveolar areas appeared to be equal.

B. Castrated female mice bearing intrasplenic ovarian transplants.—Four luteomas and 7 mixed tumors were found among the 33 castrated female mice of the A strain that showed no adhesion of the

intrasplenic ovarian graft to the left uterine horn or to the adjacent peritoneum. Among the 19 ovariectomized mice of the same strain with vascularized adhesions of the ovarian transplant, 1 luteoma was observed in a mouse that had irregular estrous cycles during the latter part of the experimental period (Table I). Unmixed granulosa-cell tumors were not noted. The ages of the intrasplenic ovarian transplants are summarized in Fig. 2. The mean age of the grafts that became tumorous was greater in the females (278 days) than in the males (236 days).

Microscopically, the luteomas were composed of polyhedral, lutein-like cells with finely granulated, acidophilic cytoplasm, and vesicular nuclei; mitoses were rare (Figs. 14, 15). The luteoma cells formed small tubular or cord-like structures surrounded by delicate connective tissue septa and were usually assembled, as in the granulosa-cell tumors, into large masses that showed incomplete encapsulation. Blood capillaries and sinusoidal spaces were observed among the tumor cells, and tissue mast cells and pigment-containing cells occasionally occurred in some areas. Groups of large, probably lutein cells, cells having a large lightly staining cytoplasm, were found frequently in the tumors.

Seven tumors in this group of experiments were of mixed type consisting of elements of both granulosa cells and luteomatous tissue (Fig. 16). Most

TABLE I: TUMORS IN OVARIES TRANSPLANTED TO CASTRATED MICE

Site of transplant	Hosts	Number of mice	Granulosa-cell	Ovarian tumors		
				Luteoma	Mixed	None
Spleen	Castrated males	21	5 (224–268 days) 2? (130, 262 days)	....	1 (225 days)	13 (75–268 days)
Spleen	Castrated females	33	....	4 (153–284 days)	7 (266–346 days)	22 (25–334 days)
Spleen (adhesion)	Castrated females	19	....	1 (262 days)	....	18 (95–346 days)
Subcutaneous	Castrated females	25	1? (243 days)	....	....	24 (61–278 days)
Subcutaneous	Intact males	12	....	....	....	12 (141–239 days)
Subcutaneous	Castrated males	9	....	....	....	9 (64–233 days)
Testis	Intact males	34	....	....	....	34 (32–370 days)

DESCRIPTION OF FIGURES 4 TO 8

FIG. 4.—Granulosa cell tumor in an intrasplenic ovarian transplant in a castrated male mouse bearing the transplant for 268 days.

FIG. 5.—Section of a granulosa cell tumor as shown in Fig. 4. Mag. × 105.

FIG. 6.—Granulosa cell tumor in an intrasplenic ovarian transplant (247 days) in a castrated male mouse, showing folliculoid structures. Mag. × 192.

FIG. 7.—Folliculoid structures in a granulosa cell tumor resembling *ovarian follicles*. This tumor developed in an intrasplenic ovarian transplant (225 days) in a castrated male mouse. Mag. × 99.

FIG. 8.—A diffuse mass of granulosa tumor cells showing mitotic figures. This tumor developed in an intrasplenic ovarian transplant (273 days) in a castrated female mouse. Mag. × 192.
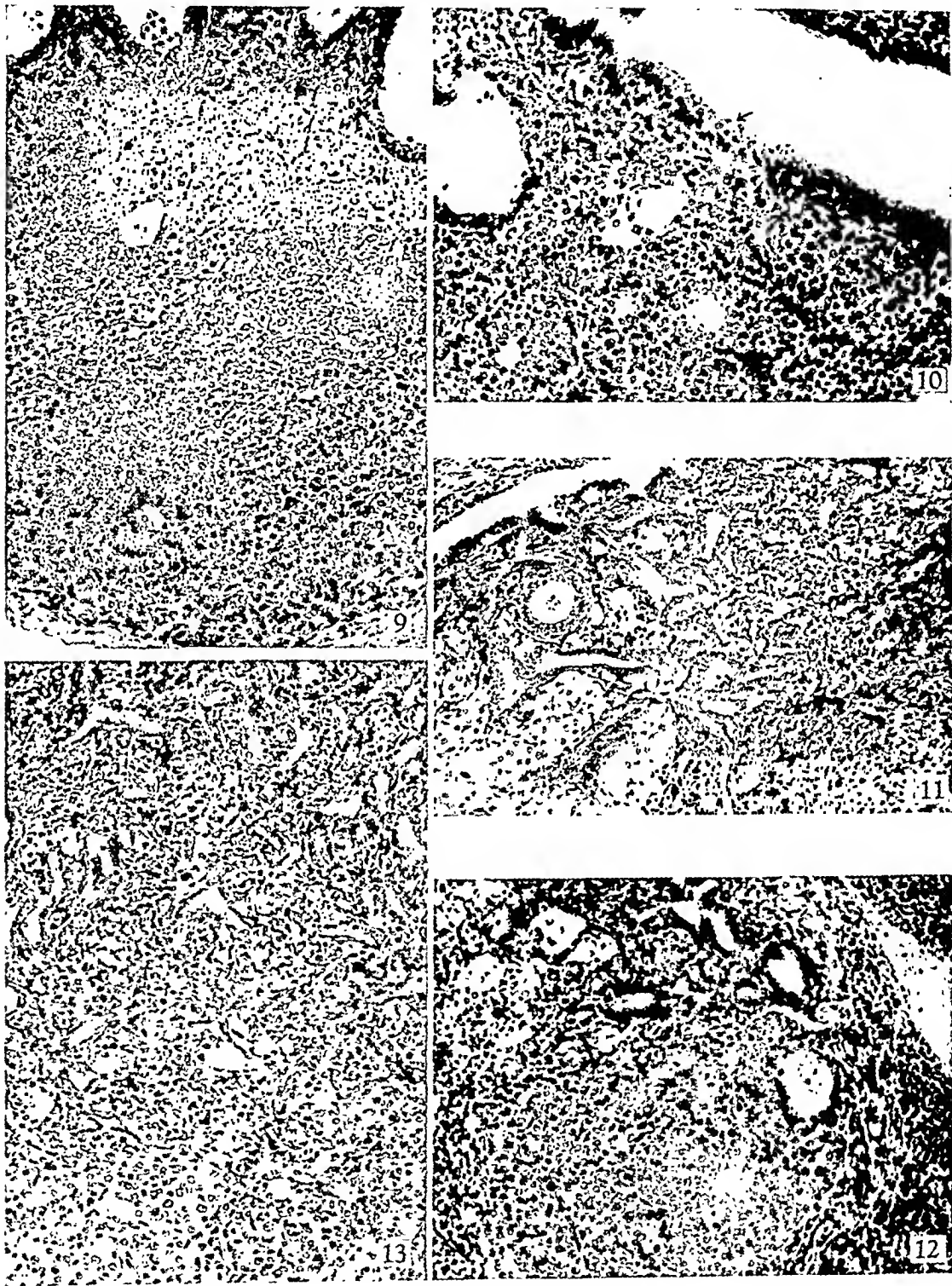
Figs. 9-13

granulosa-cell tumor masses exhibited the folliculoid pattern as described above for the castrated male mice. Transformation of granulosa cells of the tumors into luteomas appeared to take place in some of the masses of the former type (Fig. 17). Many granulosa cells showed vague cell boundaries indicating that they were becoming luteinized.

Proliferation and ingrowth of the germinal epithelium were observed in all of these tumors. The luteoma cells were sometimes in direct contact with the tubular ingrowth of the germinal epithelium and sometimes intermingled at the borders of masses of less differentiated granulosa tumor cells (Fig. 18).

Four castrated female mice that had tumors of the intrasplenic ovarian transplants showed estrous vaginal smears; 2 early and 2 in the latter part of the experiment. No estrous smears were obtained from the other 8 ovariectomized mice that had tumors in the intrasplenic ovarian transplants; their uteri were small, averaging about 37 mgm. Alveolar formation was not observed in the mammary glands of two animals that had viable intrasplenic ovarian transplants. The nuclei of the stromal cells of the endometria from the castrated females with the intrasplenic ovarian tumors were generally small, dense, and spindle-shaped, resembling those found in the uteri of castrated mice (44). The x-zone of the adrenal glands was absent and areas with compact groups of rounded or fusiform cells were found in the immediate subcapsular region of the cortex of almost all adrenals from mice of this group. The latter condition was noted only in the castrated female mice. Similar changes associated with the formation of adrenal cortical carcinoma have been described by other workers in spayed female mice of the extreme dilution strain (90). As in the castrated males, the parietal layer of the renal capsules was usually composed of low epithelial cells. The relative extent of tubular and alveolar areas in the submaxillary glands were equal, but the terminal

tubules, in most cases, essentially consisted of columnar cells. The animals with adhesion of the intrasplenic ovarian transplants had submaxillary glands of the female type.

Fourteen of the 22 intrasplenic ovarian transplants that did not become tumorous were less than 250 days old, and only one tumor was noted in a younger graft (153 days). Irregular estrous smears were obtained in 12 of the 22 castrated female mice, and the average uterine weight of these 12 female hosts was 50 mgm.

The stroma of all but one of the nontumorous ovarian grafts consisted predominantly of large lutein-like interstitial cells arranged in masses, in islands separated by sinusoids, or in cords separated by sinusoids, or ingrowing strands from the germinal epithelium. Usually these cells had a granular eosinophilic cytoplasm but sometimes the cytoplasm showed little affinity for the stains. Almost all of the grafts contained a few small or primary follicles. Large follicles were found in all but one of the grafts less than 250 days old, and were rarely noted subsequently. Hemorrhagic follicles or cysts were present in all but 2 of the grafts. The first evidence of ingrowth of the germinal epithelium was noted in an ovary that had been grafted in the spleen for 110 days. None of the other 11 grafts less than 200 days old showed epithelial ingrowths whereas 5 of the 10 grafts over 200 days of age showed ingrowths of the germinal epithelium. These epithelial ingrowths were adjacent to areas of lutein-like stromal cells, and in some places the continuity was such as to suggest the origin of the latter by differentiation from the former.

No typical corpora lutea were noted but 5 of the 12 intrasplenic ovarian grafts less than 200 days old contained structures considered to be luteinized follicles, some of which closely resembled old corpora lutea. Lutein-like cells were noted along the walls of some large follicles indicating that the granulosa cells were undergoing transformation. There was no evidence of cyclic formation of

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DESCRIPTION OF FIGURES 9 TO 13

FIG. 9.—Partial luteinization of granulosa tumor cells. This tumor developed in an intrasplenic ovarian transplant (247 days) in a castrated male mouse. Mag. × 168.

FIG. 10.—Local proliferation of germinal epithelium in an intrasplenic ovarian transplant (225 days) in a castrated male mouse. Note the mitotic activity (arrow indicated) and the close association of the germinal epithelium with granulosa tumor cells. A bursal space separated this part of transplant from the splenic tissue (above). Mag. × 178.

FIG. 11.—Tubular ingrowth from germinal epithelium in an intrasplenic ovarian transplant (225 days) in a cas-

trated male mouse. Note the intimate relationship of the primary ovocyte, lutein cells and follicle cells with the network of epithelial ingrowths. Mag. × 171.

FIG. 12.—Section showing the close associations of tubular ingrowths with granulosa tumor cells, and the transplant with the splenic tissue at right. Same tumor as illustrated in Fig. 10. Mag. × 178.

FIG. 13.—Section showing indication of transformation of granulosa tumor cells from tubular ingrowths of the germinal epithelium. This tumor developed in an intrasplenic ovarian transplant (262 days) in a castrated male mouse. Mag. × 192.
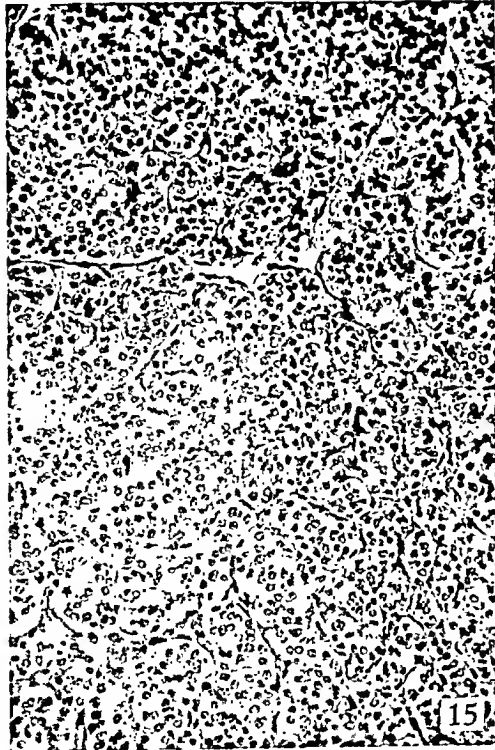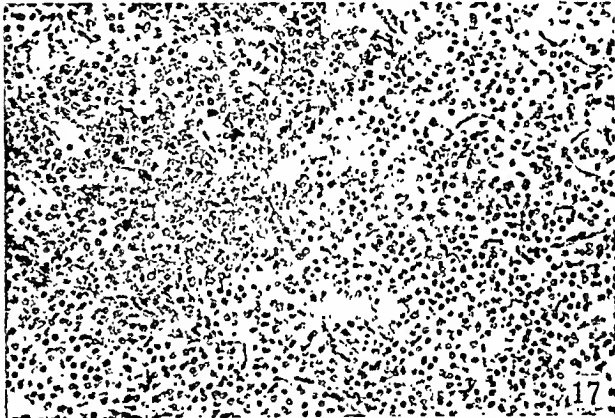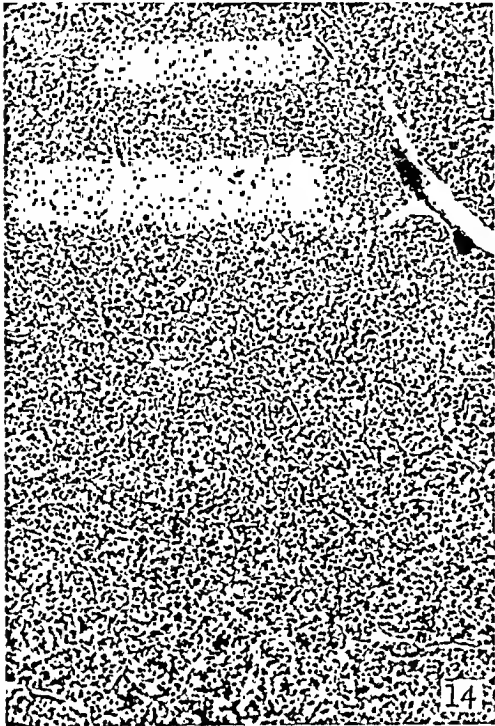
FIGS. 14-18

corpora lutea such as has been found in ovaries of intact mice. The corpora lutea-like structures were not observed in the intrasplenic grafts in the males. In this respect, in the somewhat delayed appearance of epithelial ingrowth into the stroma and of tumor formation, and in type of tumor formed, the grafts of the males and females differed appreciably.

The intrasplenic ovarian transplants in castrated female mice that had adhesion of the spleen, or graft to the adjacent body wall, uterus or kidney capsule, with one exception, were quite different from those in the mice that had no adhesion (Fig. 3). Almost all of the grafts contained a greater number of large and small ovarian follicles and corpora lutea, or follicles with completely luteinized cells (Table II). Although lutein-like stroma

TABLE II: INTRASPLENIC OVARIAN TRANSPLANTS IN CASTRATED FEMALE MICE WITH ADHESIONS TO STRUCTURES DRAINED BY CAVAL SYSTEM OF VEINS*

Age group (days)	No. of mice	Large follicles	Small follicles	Corpora lutea	Hemorrhagic cysts	Luteinized stroma	Germinal epithelium ingrowth
					Number of ovarian transplants with:		
51–100	1	1	1	0	0	1	0
101–150	1	1	1	1	0	1	0
151–200	2	2	2	1	1	2	0
201–250	3	3	3	3	0	2	0
251–300	8	6	6	1	3	5	2
301–350	4	3	3	2	1	1	0

*Studies were made on test sections removed from several different areas of the transplants.

cells were apparent in several of the grafts they made up but a small part of the stroma in most instances, a more normal fibrous stroma predominating. Only in one mouse (a 262 day old transplant) was a marked overgrowth of lutein-like cells noted; they were sufficiently anaplastic in appearance to be classified as luteomatous. One of the grafts had largely regressed (270 days) and one consisted only of stroma and large sinusoids (324 days). Ingrowth of the germinal epithelium occurred in only 2 grafts, one of which was the luteoma.

In summary, the presence of adhesions was associated with the more normal constitution of the ovarian tissues, namely, the occurrence of follicles

and corpora lutea and a low incidence of ingrowth of the germinal epithelium.

II. SUBCUTANEOUS TRANSPLANTS OF OVARIES

A. Castrated female mice bearing subcutaneous ovarian transplants.—Among the 25 castrated female mice of the A strain with ovaries transplanted subcutaneously, one small granulosa cell-like growth was observed upon histological study (Fig. 19). The mouse was the only one that was operated upon during pregnancy and carried the graft for 243 days. Masses of granulosa cells surrounded many irregular hemorrhagic cavities and mitotic figures were frequent. Lutein cells, luteinized granulosa cells and one primary follicle were also found in this transplant. The ingrowths of the germinal epithelium formed many tubular or cystic structures that merged into the tumor-like masses. No estrous cycles were noted in this mouse during the first month after the operation, but cornified vaginal smears were obtained in the latter part of the experiment. The uterus weighed 153 mgm. at autopsy.

The other 24 castrated females carried the subcutaneous ovarian transplants for periods of 61 to 278 days. Although most of them were killed 8 months after the operation, the grafts usually were not as large or as well developed as those in the spleen, and in 7 of the 24 hosts ovarian tissues could not be definitely identified. The subcutaneous, fibro-fatty tissues encroached upon the grafts except where a capsule lined by the germinal epithelium intervened. Small masses of lymphoid tissue or areas of diffuse lymphoid tissue occupied parts of the ovarian grafts and of the adjacent tissues. Most of the ovarian grafts contained primary and maturing follicles, and luteinized follicles, some with hemorrhagic centers (Fig. 20). The stroma was composed of lutein-like interstitial cells, and in 4 grafts large areas, apparently of interstitial cells or luteinized follicles, were necrotic. Degeneration of this type has been noted in the ovaries of old mice but was not observed in the intrasplenic ovarian transplants. Ingrowth of the germinal epithelium was noted in

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DESCRIPTION OF FIGURES 14 TO 18

FIG. 14.—Luteoma in an intrasplenic ovarian transplant (284 days) in a castrated female mouse. Mag. × 93.

FIG. 15.—Higher magnification of Fig. 14. Mag. × 186.

FIG. 16.—A mixed tumor consisting of granulosa cells (right) and luteoma cells (left). This tumor developed in an intrasplenic ovarian transplant (334 days) in a castrated female mouse. Mag. × 89.

FIG. 17.—Section showing indication of transformation of granulosa tumor cells into luteomatous tissue in an intrasplenic ovarian transplant (273 days) in a castrated female mouse. Mag. × 178.

FIG. 18.—Section showing intimate relationship between tubular ingrowths of the germinal epithelium and luteoma cells. Same tumor as illustrated in Fig. 17. Mag. × 93.

FIGS. 19-22

3 of the grafts in addition to the 1 that had become tumorous.

Estrous vaginal smears were obtained from these animals and the average weight of their uteri was 60 mgm. Some development of alveoli was maintained in the mammary glands. The adrenal glands had no x-zones and in the subcapsular region of the adrenal cortex there were compact groups of deeply stained cells similar to those observed in the group of castrated female mice with the intrasplenic ovarian transplants. Some cuboidal cells were found in the parietal layer of the Bowman's capsules. The submaxillary glands exhibited the female type of histological appearance with more alveolar than tubular structures and the terminal tubules were mainly composed of cuboidal cells.

B. Castrated male mice bearing subcutaneous ovarian transplants.—Nine castrated male mice of the A strain had ovaries placed in the subcutaneous tissue and 8 were killed 173 to 233 days later (Fig. 3). Ovarian tissue was not found in the sections prepared from 3 of the grafts; they consisted of cysts of the ovarian bursae or oviducts. Some ovarian tissue may have been present at levels other than those at which the sections were removed, although several sections were taken from different parts of each transplant.

The one transplant, removed 63 days after grafting, contained many large follicles, some cystic but still lined by granulosa cells, and several smaller follicles. Corpora lutea were not present. The ovary was largely surrounded by a bursa-like cavity. The germinal epithelium had not invaded the ovary. The 173 to 233 day old transplants from 5 mice contained a few large follicles or follicular cysts, some of which contained blood and a few small follicles. Some small follicles contained partially luteinized granulosa cells. The stroma consisted of large cells with a pale-staining cytoplasm and was broken up by extensive ingrowth of cells from the germinal epithelium (Fig. 21). In at least 2 of the ovarian transplants small areas of stroma composed of lutein-like cells were reminiscent of the intrasplenic transplants in the castrated mice, although in almost all instances the grafts in the subcutaneous tissue were much smaller.

Atrophy of the seminal vesicles and prostates were observed in these castrated male mice. The mammary glands showed estrogenic stimulation with some growth of alveoli; and the submaxillary glands exhibited female characteristics. Groups of deeply stained small cells occurred in the subcapsular region of the adrenal cortex and no x-zone was present. In some Bowman's capsules the parietal lamina consisted partially of cuboidal epithelial cells.

C. Intact male mice bearing subcutaneous ovarian transplants.—The ovarian transplants of 8 of the 12 intact male mice bearing subcutaneous ovarian grafts for 140 to 239 days were examined histologically (Fig. 3). All of the grafts were small and were partially surrounded by bursa-like cavities. They contained few to many small and rarely a medium sized follicle. The stroma was composed primarily of compact fibroblast-like cells and "wheel cells" with small nuclei containing several aggregates of chromatin; stroma such as is found in the ovaries of hypophysectomized mice. A few "brown" cells and rarely a large cell with a clear cytoplasm were also observed in the stroma. Although the germinal epithelium was thick in many places, definite tubular ingrowths into the transplants were not noted. The small size of the grafted ovaries, absence of large follicles or corpora lutea, and the appearance of the stromal cells all indicated that the ovaries were but slightly stimulated by the gonadotrophic hormones of the hosts. In general, there were no appreciable effects of ovarian hormones in the male genital organs, adrenals, kidneys, and submaxillary glands.

III. INTRATESTICULAR TRANSPLANTS OF OVARIES

Thirty-four mice, 13 of the A strain and 21 hybrids (AC₃, AC₅), carried intratesticular ovarian grafts for 32 to 355 days. Twenty-four of the mice were killed when the grafts were over 250 days old, and 13 grafts were over 350 days old (Fig. 3).

The two grafts removed when less than 50 days old were small, were surrounded by bursa-like spaces, and contained chiefly follicles at several

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DESCRIPTION OF FIGURES 19 TO 22

Fig. 19.—Granulosa cell-like growth, with evidence of epithelial ingrowth, in a subcutaneous ovarian transplant (243 days) in a castrated female mouse. Mag. × 42.

Fig. 20.—Subcutaneous ovarian transplant (242 days) in a castrated female mouse showing ovocyte, tubular ingrowth, hemorrhagic cyst, interstitial cell stroma, and necrotic areas. Mag. × 100.

Fig. 21.—Subcutaneous ovarian transplant (233 days) in a castrated male mouse showing follicle, tubular ingrowth, and interstitial cell stroma. Mag. × 100.

Fig. 22.—Intratesticular ovarian transplant (351 days) in an intact male mouse showing follicular cysts, hemorrhagic cysts, tubular ingrowth, and interstitial cell stroma. Mag. × 42.

stages of development. The older grafts were similar to one another in most respects and will not be described separately. All of the grafts, except one that was composed entirely of interstitial cells (315 days old), were covered in part by a bursa-like space or spaces lined by columnar or cuboidal epithelium, apparently the persisting germinal epithelium. Epithelial extension from the surrounding bursa occurred in most of the grafts over 250 days old and tended to increase with advancing age (Fig. 22). The epithelial extensions grew only into the ovarian tissue and not out into the testis although both sides of the bursa were lined by cells that appeared to be identical. The germinal epithelial cells did not grow into the testicular stroma. The epithelial ingrowths resembled those observed in grafts at other sites in castrated male and female hosts. In several grafts the tubular ingrowths of germinal epithelium had become cystic and sometimes contained blood. The cells lining the cysts sometimes contained hemosiderin, and in places resembled interstitial cells and macrophages.

Most of the grafts contained several follicles of different sizes. A few follicles contained blood and the cells lining the walls were somewhat luteal. No corpora lutea or completely luteinized follicles were found.

The greater part of most of the grafts consisted of a stroma composed mainly of large, lightly stained cells with definite cell membranes, and of large cells containing a brownish pigment scattered among small blood vessels and sinusoids, and fibroblastic elements. Such tissue has been classed as interstitial in the ovarian grafts at other sites.

A number of the ovarian transplants, especially the older ones, contained small areas of cord-like or solid follicle-like structures of granulosa cells somewhat reminiscent of the granulosa-cell tumors. The scarcity of mitotic figures, the small size of the areas, and well organized stroma led to the impression that they should not be designated as tumorous. The impression was gained, however, that ovarian grafts in the testes might occasionally become tumorous.

The mammary glands in the male hosts with ovarian transplants showed a partially developed duct system, although kidneys, submaxillary glands, and other genital organs showed normal male characteristics. No x-zone was present in the adrenals and the growth of compact small cells in the subcapsular region of the adrenal cortex with occasional mitotic figures was found in the hybrid male mice. In mice of the A strain, however, this condition was observed in the castrated females and in castrated males bearing subcutaneous ovarian grafts.

DISCUSSION

I. Pathogenesis of the Ovarian Tumors

The high incidence of granulosa-cell tumors in women at or just preceding the menopause has been noted by several workers (15, 71, 80, 84, 87, 88), and the amenorrhea that was often ascribed to "the menopause" is now considered by Dockerty (15) as the first symptom of a granulosa-cell tumor. In presenting a case of granulosa-cell tumor in a 51 year old woman, Voigt (89) inferred that an overproduction of the gonad-stimulating hormone after the ovaries lost their inhibiting effect upon the anterior pituitary, might play a part in the formation of the tumor. Recently, Biskind and Biskind (4) reported three granulosa-cell tumors in rats' ovaries transplanted into the spleen and they suggested that the development of the tumor is due to the inactivation of ovarian estrogen by the liver, allowing the protracted stimulation of the pituitary to act on the ovary. These intrasplenic ovarian growths, however, have been questioned as "true blastomas" (80).

The present experiments provide evidence that an endocrine imbalance between the pituitary gonadotrophins and ovarian hormones is responsible for the development of granulosa-cell tumors and luteomas in mice. The increase of endogenous gonadotrophic hormones in castrated mice is manifested by the rapid growth of the ovaries and by the formation of hemorrhagic follicles shortly following the intrasplenic transplantations. The formation of hemorrhagic follicles (blood points), as Zondek (92) has pointed out, is a most conspicuous and impressive reaction in the immature mouse's ovary to the administration of anterior pituitary hormones. Furthermore, the increase of intrinsic gonadotrophins subsequent to castration has been shown by bioassays of urine, blood and hypophysis and in experimental parabiosis of intact with castrated or roentgen-rayed animals (20, 21, 23, 28, 54). Castrated female mice carrying intrasplenic ovarian transplants, but with vascularized adhesions that permitted ovarian hormones to bypass the hepatic portal circulation, showed estrous vaginal smears throughout the course of the experiment and acquired no ovarian tumors.. Ovarian tumors have not occurred in the intrasplenic transplants of ovaries in unilaterally gonadectomized male and female mice (unpublished data). The only female mouse that had a granulosa cell tumor-like growth in the subcutaneously transplanted ovary was operated upon in pregnancy

and no estrous cycles were noted during the early part of the experimental period. All the other castrated male and female mice bearing ovarian transplants at sites other than those drained by the hepatic portal system, e.g., subcutaneous or intratesticular grafts, had no ovarian tumors. It is not improbable that some of these grafts in the castrated mice might have become tumorous had they been observed at later dates.

It is of special interest that unmixed granulosa cell tumors developed in male, and luteomas in female, mice carrying the intrasplenic ovarian transplants. This may be due to the physiological difference in the pituitaries of males and females; such differences have been observed with rats (13, 66). The pituitary gland of males apparently produces less luteinizing hormone than that of the females. The luteinization of tumor cells and the transformation of the granulosa-like cells into luteomas indicate that the neoplastic growths are under the influence of the pituitary gonadotrophic hormones.

It seems possible that roentgen ray treatment of the ovaries in rats and mice may induce a similar endocrine imbalance. Levine and Witschi (49) reported that normal female rats showed constant estrus when placed in parabiosis with irradiated female rats, and the castration-cells were found in the pituitaries of the irradiated rats. Experimental induction of ovarian tumors in mice subsequent to roentgen ray irradiation has been reported by several workers. Because the histological structure of granulosa-cell tumors and the luteomas appearing in the ovaries grafted into the spleen are similar, if not identical, to the ovarian tumors induced with roentgen rays[3], it seems that the same mechanism, namely gonadotrophic overaction, is responsible for the tumors under both circumstances.

In the present experiments ovarian tumors occurred in mice of the A strain. The question of strain limitation in formation of the tumors in mice may be raised. Results of experiments in progress show rapid growth of the intrasplenic ovarian transplants in mice of several strains. Lipschütz and his collaborators (52) mentioned that species differences and the duration of the experiment may account for failure to obtain malignant ovarian growths in guinea pigs. Only a few of their animals carried intrasplenic ovarian grafts for more than 1 year. However, most of the ovarian tumors in the experiments with mice developed 7 months after the transplantation.

[3] Dr. H. S. Kaplan was kind enough to permit examination of some of his unpublished material of roentgen-ray induced ovarian tumors in mice.

One testicular tumor "bearing a striking resemblance to the granulosa-cell tumor of the ovary" and with certain portions showing arrhenoblastoma-like structures developed in an intrasplenic testicular transplant in a castrated male rat (5). Intrasplenic testicular transplants in castrated male and female mice have not become tumorous (51). The higher temperature in the abdominal cavity may prevent the development of testicular transplants (59).

II. Histogenesis of the Ovarian Tumors

Although the development of granulosa-cell tumors and luteomas has been studied by many investigators, the histogenesis of these tumors remains uncertain. The main controversial opinions on the histogenesis of granulosa-cell tumors reflect to a great extent the conflicting concepts of the development of definitive germ cells in the ovary. An adequate study of the histogenesis of the ovarian tumors has often been difficult because the comparatively large size of these growths precluded a systematic study of serial sections and frequently only late stages were obtained.

The histogenesis of granulosa-cell tumors has been explained by three major theories: (A) origin from differentiated granulosa cells, (B) origin from embryonic rests, and (C) origin from mesenchyme. Histological study of the present material suggests another view of the genesis of ovarian tumors, namely, that the tumors arise from the proliferation and ingrowth of germinal epithelium. The three theories may be briefly summarized as follows:

(A) Origin from differentiated granulosa cells. Schröder (76) designated the term "folliculoma" for some granulosa-cell tumors and stated that the tumors arose from the follicular epithelium. Robinson (72) reported that ova were present in the folliculoid spaces, and that the follicle cells could undergo structural and quantitative variations of the widest range and could give rise to benign and malignant tumors. He contended that the folliculoid structures in the tumors were not the result of a process of "liquefaction" or of "degeneration", not even of a possible "reversion to type" by the granulosa cells, but were the remains of ovarian follicles. Moreover, he believed that in man there is no postnatal ovogenesis from the surface epithelium of the ovary. Dockerty and MacCarty (16) described one granulosa-cell neoplasm and supported the view that the tumor arose from the follicle cells. It has been reported that in mice roentgen irradiation destroyed ova, but left the epithelium of small follicles intact (12, 29, 85) and that granulosa cell tumors originated from a proliferation of the persisting follicle cells. Others

have maintained that the ovarian tumors produced in irradiated mice were derived from the undifferentiated parenchymal cells of the ovary, and that the granulosa cells of the graafian follicle played no role (34). They observed, however, that the theca interna cells might participate with the parenchymal cells in the early postirradiation proliferation and luteinization.

(B) Origin from embryonic rests. According to Meyer (57, 58) the tumor cells originate from redundant granulosa cells that dip down into the ovarian substance from the germinal epithelium during the formative period and remain undifferentiated. These embryonic rests under certain unknown conditions become granulosa-cell tumors. Meyer based his theory upon an isolated finding of small masses of granulosa-cells in the ovary of a 45 year old woman, and assumed that such cells were situated chiefly in the medullary portion of the ovary. He contended that granulosa cell tumors cannot arise from epithelial cells of the true follicle because the life of the well differentiated granulosa cells is dependent upon living ova, and the majority of the tumors occur in old women in whom the ovaries no longer contain follicles. Te Linde (84) described one small granulosa-cell tumor located in the medulla near the hilus of the ovary, and assumed that the only possible origin of the tumor was an embryonic granulosa rest. Similar opinions have been expressed by others (32, 62).

(C) Origin from mesenchyme. Fischel (27) suggested that the ovarian stroma and follicular cells are derived from the ovarian mesenchyme, and the primitive mesenchymal cells could assume epithelial properties under the influence of the germ cells. He stated that the germ cells migrate into the gonad from the primordial gut during early fetal life, and the germinal epithelium, therefore, did not give rise to ova, follicle cells, or connective tissue, but existed throughout life merely as the peritoneal covering of the ovary. According to Fischel's theory, tumor cells of both granulosa and theca types arise through metamorphosis of the ovarian mesenchyme. Schiller (73, 74) supported this theory and stated that the transformation of granulosa-cell tumors from an immature trabecular "form" into a mature folliculoid "form" resulted from the transformation of connective tissue-like cells into the epithelial tumor cells. This mesenchymal theory has been used as a working hypothesis by several workers (37, 63, 64, 89). A participation in the histogenesis of granulosa-cell tumors also has been suggested (87).

The present experiments provided evidences that the germinal epithelium of the engrafted mouse's ovary proliferates and gives rise to ova and follicle cells to form primary ovarian follicles. The proliferation and ingrowth of the germinal epithelium were observed in all ovarian transplants that developed into tumors and in many of the nontumorous grafts in the intrasplenic, intratesticular, and subcutaneous sites. Local proliferation of the germinal epithelium with evidence of mitotic activity occurred frequently in the tumors. The intermingling of the invaginated epithelial cells with the cells of the smaller granulosa-cell tumors and the apparent transformation of the epithelial cells, with ellipsoidal nuclei and scanty cytoplasm, into the tumor cells indicated the genesis of granulosa-cell tumors from the germinal epithelium.

The ingrowths of germinal epithelium formed groups or networks of tubules or cysts. In some instances nuclei of the epithelial cells that lined the tubules appeared to become vesicular and the whole mass of epithelial ingrowth resembled the folliculoid formations. More often, however, the folliculoid structure seemed to form within small masses of diffuse tumor cells by subsequent liquefaction of some of the centrally placed cells. Schiller (73, 74) appeared to have noted transformations of this type although he did not associate the tumor formation with the activity of the germinal epithelium in his materials. Many of the follicle-like structures contained a few degenerating cells and sometimes blood cells or coagulated fluid, but no ovocytes were found in the folliculoid spaces in the present materials. Small ovarian follicles were rarely encountered at the periphery of ovarian transplants that developed into tumors. In the nontumorous ovarian transplants the number of ovarian follicles was generally decreased in proportion to the duration of the graft. There was no indication that the granulosa tumor cells were derived from the well differentiated granulosa or theca cells of the persisting ovarian follicles.

The observations made in the present study, as well as the histological examinations of ovaries of mice in other experiments in our laboratories, have not revealed embryonic rests of granulosa cells in the ovaries. Our experimental results do not support the assumption that such an embryonic rest of granulosa cells is a prerequisite condition for tumor formation. The tumors did not appear to develop in the medullary portion of the engrafted ovary but arose in conjunction with the germinal epithelium or in conjunction with ingrowths from the germinal epithelium. The proliferation and ingrowth of the germinal epithelium were not restricted to any particular region of the ovary.

Despite the discrepancy regarding the histo-

genesis of granulosa-cell tumors, pathologists agree that some ovarian tumors, *e.g.*, cystadenomas and adenocarcinomas, are derived from the germinal epithelium (32, 56, 72). The ingrowth of the germinal epithelium in roentgen-rayed ovaries of immature and mature mice, and rats has been described (18, 61, 66). Other workers (12, 29, 85) observed "tubular adenomas," but did not report a relationship between the invaginated epithelial cells and the granulosa-cell tumors and luteomas. However, the so-called tubular adenomas in roentgen-rayed ovaries of mice differ from tubular adenomas in the ovaries of old rats (22).

The fundamental issue in the histogenesis of granulosa-cell tumors is more closely associated with the development of ovarian follicle cells than the germ cells, although they have an intimate relationship. Allen (1) called attention to the cyclical production of new germ cells from the germinal epithelium during each estrous cycle in the adult female mouse. This observation on rhythmic activity of the germinal epithelium in mature female mice has been substantiated by several other workers (8, 9). Recently, Long (53) reported the growth *in vitro* of ovarian germinal epithelium from the adult mouse's ovary and observed the formation of primary follicles from the epithelial cells. The origin of definitive ova from the germinal epithelium in the ovaries of adult rats has also been studied (11, 24, 39, 48). Hargitt (39) described the production of ova, follicle cells, and interstitial cells from the germinal epithelium in adult rats' ovaries, and in old and senile rats the germinal epithelium continues to be active, especially in producing invaginated cords of cells, but frequently they are anovular. In an extensive investigation, Evans and Swezy (24) found that ovogenesis in the rat, guinea pig, dog, cat and man occurs throughout the whole period of sexual life in a rhythm fundamentally related to the ovulation cycle. The ova and follicle cells arise by proliferations from the germinal epithelium in the form of invaginations or cords. Hartman (41) and Everett (25) reported that in the adult opossum the germinal epithelium near the region of the hilus is more active in producing new ova; and Papanicolaou (65) suggested the increased activity in the hilus of the guinea pig's ovary was due to a greater supply of blood. Early studies on the continual proliferation of germ cells from the germinal epithelium have been reviewed (83). There are many reports supporting the view that the germinal epithelium is active in producing ova in adult mammalian ovaries: the bat (38), the guinea pig (7, 40, 75), the rabbit (19), the ground squirrel (70), the prairie dog (82) and the dog (2).

Luteomas appear to arise also from the tubular ingrowths of the germinal epithelium, although there are instances showing that the tumors may arise indirectly by transformation from granulosa tumor cells. The cells in a granulosa-cell tumor may partially luteinize, and large lutein cells are present among the luteomatous cells. The possibility that luteomas originate from proliferation of the lutein cells, as suggested by Butterworth (12), was not confirmed by our observations. Luteinization of the granulosa tumor cells has been reported (43, 63, 86). The present studies reveal that granulosa-cell tumors and luteomas are derived from the germinal epithelium. The general concept of ovarian epithelial tumors originated from the invading germinal epithelium has been postulated by Goodall (36) and others.

SUMMARY AND CONCLUSIONS

1. Five granulosa-cell tumors, 2 pretumorous lesions and 1 mixed tumor (granulosa and luteoma cells) were found among 21 castrated male mice of the A strain bearing intrasplenic ovarian transplants. Five luteomas and 7 mixed tumors occurred in intrasplenic ovarian transplants in 52 castrated female mice of the same strain. Most of the ovarian tumors developed after 7 months from time of operation.

2. Castrated female mice bearing intrasplenic ovarian transplants with vascularized adhesions that permitted drainage through other than the hepatic portal system showed estrous vaginal smears throughout the course of the experiment and acquired no ovarian tumors. The only female mouse that had a granulosa cell tumor-like growth in the subcutaneously transplanted ovary was operated upon during pregnancy and no estrous cycles were noted during the early part of the experimental period. All the other intact or castrated male and female mice bearing ovarian transplants in subcutaneous or testicular sites had no ovarian tumors.

3. The intrasplenic ovarian transplants in the gonadectomized hosts attained larger size and more frequently showed extensive tubular ingrowth of germinal epithelium than did those at other sites.

4. The germinal epithelium in the ovarian transplants give rise to the primary ovocytes and follicle cells. The granulosa-cell tumors appear to be derived from the ingrowth of germinal epithelial cells.

5. The luteomas develop either directly from the ingrowth of the germinal epithelium or indirectly from granulosa-cell tumors.

6. Ovarian hormones produced by the intrasplenic ovarian transplants are inactivated by the liver before entering the systemic circulation, while the hypophysis, under such conditions as physiological castration, produces larger amounts of gonadotrophic hormones to further stimulate the ovarian transplants. The endocrine imbalance so established is assumed to be responsible for the genesis of the ovarian tumors in the present experiments and possibly also in the ovaries of roentgen ray irradiated mice.

7. The influence of ovarian transplants on the accessory genital tissues and other organs in mice was described.

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Dosage of Carcinogen as a Modifying Factor in Evaluating Experimental Procedures Expected to Influence Formation of Skin Tumors*

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Although a particular experimental procedure might augment or inhibit tumor incidence when the carcinogenic stimulus employed to induce tumors is mild or moderate, it is possible that the effect might not be evident when higher dosages of carcinogen are utilized. This possibility was suggested by negative results of investigations in which only large dosages of carcinogen were employed, and by a few studies in which the effects of both small and large dosages of carcinogen were compared. At a meeting at Madison, Wisconsin, in 1937, Kreyberg referred to massive amounts of carcinogen as "steam-roller" doses, implying that they were large enough to mask or obliterate the effects obtainable by the same experimental procedure but with smaller doses of carcinogen.

In evaluating carcinogenesis, at least two aspects of tumor formation may be considered: (a) the incidence of tumors, and (b) the time of appearance of the tumors. Although a large dose of carcinogen may induce up to 100 per cent tumors in both a control and experimental group of animals, it is still possible that differences in the time of appearance of the tumors may be demonstrated.

In experiments reported from this laboratory it was shown that calorie-restricted diets inhibited the formation of benzpyrene-induced skin tumors in mice (9) and that fat-enriched diets (10) enhanced the formation of such tumors. In those experiments the skin tumors were induced by application of moderate dosages of carcinogen. The tumor response was controlled by terminating the application of carcinogen before any appreciable number of skin tumors had appeared, the dosage being gauged to produce tumors in between 40 and 70 per cent of the mice in the control (*ad-libitum*, low-fat diet) groups. The influence of calorie restriction or fat enrichment of the diet was demonstrated principally by the effect on the incidence,

or number of mice developing tumors. Only secondary attention was given the rate of appearance of the tumors, although it was pointed out that in those groups in which fewer tumors arose, these tumors tended to appear, on the average, at a later time.

This report is concerned with experiments designed to test whether or not the inhibiting effect of calorie restriction and the augmenting effect of fat enrichment of the diet, observed when moderate dosages of carcinogen were utilized, would be obliterated by the use of larger amounts of carcinogen.

METHODS

In both of the studies reported here 4 groups, each of about 50 dba strain male mice, were utilized. The mice were born within a 4 week period and at weaning the litter mates were distributed between the 4 groups of the experiment. When the mice were about 9 weeks of age they were transferred from the stock diet of Purina dog chow checkers to their respective experimental diets.

In each study, 2 of the 4 groups were fed a control ration consisting of 1.4 parts of Purina dog chow meal, 0.9 parts of skimmed milk powder, and 1.9 parts of cornstarch. This ration permits mice to grow normally. It contains 15 per cent protein, 2 per cent fat, and 70 per cent carbohydrate, along with adequate vitamins and minerals. The other 2 groups of the particular study were fed the experimental diets (calorie-restricted or fat-enriched) which are described later in the experimental sections.

Application of carcinogen (one drop of a 0.3 per cent solution of 3,4 benzpyrene in benzene, applied by means of a dropper to the skin of the interscapular area) was begun 4 weeks after the diets had been initiated. In each study the mice of 1 control group and of 1 experimental group were given 19 applications in 9 weeks, while the mice of the other control group and experimental group were given 50 applications in 18 weeks. Thus the former 2 groups were considered to have received

a moderate dosage of carcinogen, and the latter 2 groups a large dosage. This assumption was confirmed by the tumor response.

The housing, feeding, care, inspection, and observation of the animals have been described in previous publications (9, 10). The animals were examined for skin tumors and weighed in general, at 2 week intervals except during the period when the tumors were appearing at a rapid rate; during this time the mice were inspected once a week. The percentage of mice with tumors was calculated on the basis of the number of animals alive at the time the first tumor appeared in the experiment (effective total). The total number of mice with tumors and time of appearance of tumors refer to the first tumor appearing in each mouse. In most mice the tumors were recognized initially as papillomas, in others as carcinomas; these were all grouped and reported as skin tumors.

EXPERIMENTAL

THE EFFECT OF DOSAGE OF CARCINOGEN ON THE INHIBITING ACTION OF A CALORIE-RESTRICTED DIET ON THE FORMATION OF INDUCED SKIN TUMORS

In this experiment the 2 groups (A0, A60) that received the control ration (as described in Methods) were given 4.2 gm. per mouse daily. The mice of the 2 groups given the calorie-restricted ration (A5, A65) were given 2.3 gm. of a ration consisting of the same amount of Purina dog chow meal and skimmed milk powder, but without the added cornstarch. The control mice ate, on the average, 3.8 to 4.1 gm. daily, while the calorie-restricted mice ate all of the food given them, namely 2.3 gm. One may consider that the mice of all 4 groups consumed relatively equal quantities of protein, vitamins, and minerals and that the mice on the restricted diet were restricted in carbohydrate only.

Control group A0 and calorie-restricted group A5 received the moderate dose of carcinogen while control group A60 and calorie-restricted group A65 were given the large dose of carcinogen. At the initiation of the experimental diets, 4 weeks before the first application of carcinogen, the mice of each of the 4 groups weighed 22 gm., on the average. From the beginning of the application of carcinogen until the end of the experiment, the mice of the calorie-restricted groups maintained an average weight of 21 gm. Mice of the *ad-libitum* control groups had grown to 27 gm. on the average when the application of carcinogen was begun. During the following 16 weeks the control mice receiving the moderate dosage (A0) grew to an average weight of 34 gm. and very nearly maintained this weight during the subsequent course of the experiment, while the mice given the large dosage (A60) grew somewhat more slowly reaching only 31 gm. average weight (due probably to the greater toxicity of the larger dosage of carcinogen).

Groups A60 and A65 (large dosage) were terminated at 38 weeks after the first application, at which time none of the A60 mice were alive and no new tumors had appeared in A65 for 8 weeks. Groups A0 and A5 (moderate dosage) were terminated at 60 weeks since by this time very few tumors were appearing. The curves of tumor formation are presented in Fig. 1 and salient data and statistics in Table I.
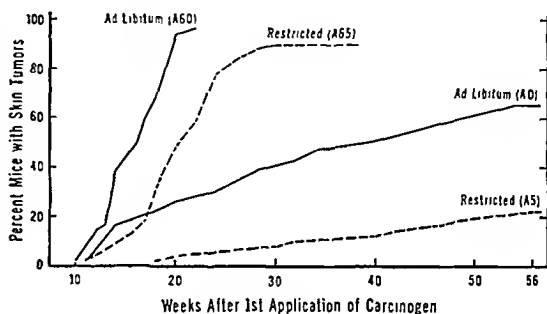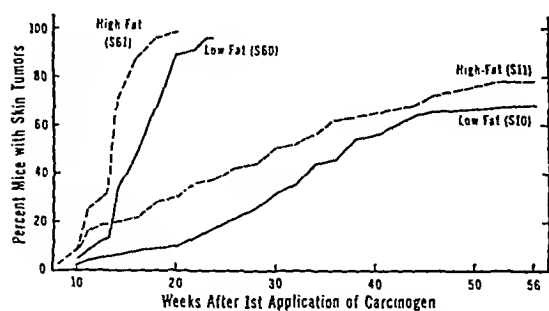

FIG. 1.—Formation of skin tumors induced by 3, 4-benzpyrene. Curves showing effect of caloric restriction when moderate dosage (A0, A5) and large dosage (A60, A65) of carcinogen where utilized.

Effect on incidence of tumors.—The striking effect of caloric restriction on the incidence of mice developing skin tumors observed when the dosage of carcinogen was moderate (65 per cent in the control A0, as compared with 22 per cent in the calorie-restricted group A5) was obliterated by the higher dosage (96 per cent in the control A60, and 90 per cent in the calorie-restricted group A65). However, a residuum of the effect on incidence is suggested by the fact that 3 of the mice of the calorie-restricted group, A65, had not developed skin tumors 38 weeks after the first application of carcinogen—8 weeks after the last tumor had formed in this group and 16 weeks after all the mice of the *ad libitum* control group, A60, had either developed skin tumors or died.

Effect on time of appearance of tumors.—The use of the high dosage of carcinogen yielded results which emphasized the effect of calorie-restriction on the mean time of appearance of tumors. While the mean times of appearance were shortened, and even the actual magnitude of the difference was decreased by the employment of a large dosage of carcinogen, the difference has greater statistical significance because the range of time over which

the tumors appeared was markedly decreased. The question of statistical significance as related to evaluating the effects of caloric restriction will be considered more fully in the discussion.

The data suggest that with moderate dosages of carcinogen the effect of caloric restriction on tumor formation is revealed principally through a reduction in the incidence of tumors, while with large dosages of carcinogen principally through the delay or postponement in appearance of the tumors.

the control group S60 and the fat-enriched group S61 were given the large dose of carcinogen.

At the first application of carcinogen, the average weight of the mice of each group was 25 gm. During the following 16 weeks the mice of the groups receiving the moderate dosage (S10 and S11) grew to an average of 32 gm. and very nearly maintained this level during the course of the experiment. The increased toxicity of the large dosage was manifested in the slightly depressed growth of the mice given 50 applications of car-

TABLE I: THE EFFECTS OF CALORIC RESTRICTION AS INFLUENCED BY DOSAGE OF CARCINOGEN

0.3% Benzpyrene in benzene	Group	Number of mice	Mice alive and free of tumors at end of experiment	Mice with tumors No.	Mice with tumors %	Time of appearance of skin tumors (weeks) Mean	Variance*	Difference between means	Significance of difference t	Significance of difference P
Moderate dosage, 19 applications	A0: Adlibitum	49	4	32	65	28.3±2.4	178	9.7±4.6	2.1	less than 5%
	A5: Caloric restricted	50	26	11	22	38.0±4.0	172			
Large dosage, 50 applications	A60: Adlibitum	49	0	47	96	16.3±0.5	10.3	4.4±0.81	5.5	less than 0.1%
	A65: Caloric restricted	48	3	43	90	20.7±0.7	18.3			

*In Tables I and II, the "variance" is a measure of the dispersion of the times of appearance (the square root of the variance is the standard deviation). The greater the tendency for the individual values to gather about the mean time of appearance, the smaller the variance. "t" is the ratio of the difference between the two means being compared to an estimate of the standard error of the difference; the standard error being a function of the variances of the two groups. "P" is the probability that the value of "t" could have occurred on the basis of chance alone—i.e. if there had been no real difference between the groups due to the experimental procedure. It is obtained from prepared Tables which give the relation between "t" and "P." The greater is t, the smaller is P and hence the less the probability that the observed difference is not real. A value of P less than 5% is generally considered to indicate "statistical significance" of the difference. These terms and their implications are considered further in the discussion; details of the test and its application may be found in any recent standard text on statistics (4).

EFFECT OF DOSAGE OF CARCINOGEN ON THE AUGMENTING ACTION OF A FAT-ENRICHED DIET ON THE FORMATION OF INDUCED SKIN TUMORS

The two groups (S10, S60) that received the control ration (as described in METHODS) were given 4.2 gm. per mouse daily. The mice of the 2 groups (S11, S61) fed the fat-enriched diet were given a ration containing the same amount of Purina dog chow meal and skimmed milk powder, but 0.9 gm. of fat[1] were substituted for 1.9 gm. of cornstarch. The resultant ration contained 19 per cent protein, 31 per cent fat, and 38 per cent carbohydrate. The control mice ate, on the average, 3.8 to 4.2 gm. daily, while the mice on the fat-enriched diet ate from 2.9 gm. to all of the 3.2 gm. given them. For practical purposes one may consider that the mice of all 4 groups ate approximately equal amounts of protein, vitamins, and minerals.

Control group S10 and fat-enriched group S11 received the moderate dose of carcinogen while

cinogen (S60 and S61); the mice of these 2 groups reached a maximum average weight of 30 gm.

The experimental groups receiving the large amount of carcinogen (S60, S61) were terminated at 31 weeks after the first application of carcinogen, and those given the moderate dosage (S10, S11) were terminated at 56 weeks, since there were very few mice without tumors at these times. The curves of tumor formation are presented in Fig. 2 and summarized data are given in Table II.

Effect on incidence of tumors.—In the two groups to which the moderate dosage of carcinogen was applied, the incidence of mice with tumors was 68 per cent in the control S10 group compared with 78 per cent in the S11 group consuming the fat-enriched diet. This difference, while small and not in itself of statistical significance, is reproducible (10) having been obtained repeatedly in our laboratory when dosages of comparable order were employed.

On the other hand, the application of the larger amount of carcinogen obliterated the slight augmentation of incidence of tumors, 96 per cent and 98 per cent occurring in groups S60 and S61 respectively.

[1]Kremit—partially hydrogenated cottonseed oil, furnished generously by Armour and Company, Chicago.

FIG. 2.—Formation of skin tumors induced by 3, 4-benzpyrene. Curves showing effect of a fat enriched diet when moderate dosage (S10, S11) and large dosage (S60, S61) of carcinogen where utilized.

Effect on time of appearance of tumors.—The difference in mean time of appearance of tumors, between groups S10 and S11 (moderate amount of carcinogen) was not of statistically significant magnitude, although it is in the direction expected

and because of the comparable results of other workers (5, 11). This experiment (S60 and S61) is therefore the first single experiment with induced skin tumors, in which using groups of only 50 mice we have obtained a difference that in itself, on the basis of statistical criteria and independent of the results of other experiments, proves the augmenting effect of a fat-enriched diet.

The evaluation of these results (effect of fat-enriched diet) is in agreement with that of the previous experiment (effect of calorie-restricted diet). With moderate dosages of carcinogen these dietary changes revealed their effects on the formation of induced skin tumors principally through changes in incidence, while with large dosages the effects were shown mainly through changes in the mean time of appearance (induction time).

DISCUSSION

In the present experiments, the large dosage of carcinogen, by producing a very high percentage

TABLE II: The Effects of a Fat-enriched Diet as Influenced by Dosage of Carcinogen

0.3% Benzpyrene in benzene	Group	Number of mice	Mice alive and free of tumors at end of experiment	Mice with tumors No.	Mice with tumors %	Time of appearance of skin tumors (weeks) Mean	Time of appearance of skin tumors (weeks) Variance*	Difference between means	Significance* of difference t	Significance* of difference P
Moderate dosage, 19 applications	S10: Low fat	50	3	34	68	31.4±1.8	105	4.6±2.8	1.6	greater than 5%
	S11: Fat-enriched	50	7	39	78	26.8±2.1	176			
Large dosage, 50 applications	S60: Low fat	45	1	43	96	16.6±0.5	11.4	2.8±0.63	4.4	less than 0.1%
	S61: Fat-enriched	48	0	47	98	13.8±0.4	6.0			

*See footnote, Table 1.

from similar experiments previously reported by us. In the group developing more tumors the growths occurred at an earlier average time.

More definitively, when using the larger amounts of carcinogen, the group given the high-fat diet (S61) developed tumors at a significantly (statistically) earlier time than the group consuming the control ration. This difference in time of appearance, 2.8 weeks, is significant even though relatively small. The range of time of appearance of the tumors was abbreviated by use of the large dosage of carcinogen to the extent that even a difference of about one-half that actually obtained would have statistical significance.

This latter point is of interest since in no single previous experiment on the effect of a fat-enriched diet on skin tumor formation have we obtained a difference of statistically significant order (and hence acceptable as reliable evidence). Existence of this so called "fat-effect" was accepted because of the reproducibility of the augmenting effect,

of tumors in both control and experimental groups, obliterated the differences in *incidence* of tumors. On the other hand, the large dosage of carcinogen actually emphasized the differences in *time of appearance* of tumors. The ability to demonstrate this depended on greater statistical validity rather than on increases in the magnitude of the differences. The increased statistical significance arose from the fact that in the mice treated with the large dosage, the tumors formed within a very much shorter span of time than did the tumors of the mice treated with the smaller amount of carcinogen. The interpretation will be accepted by those with an understanding of statistics; for others, a brief discussion of the principles involved is given. This is followed by a general discussion.

Statistical.—Throughout this report the term "significance" is employed only in the statistical sense—*i.e.* it is not necessarily related to the biological importance of the effects observed. Most statistical "tests of significance" actually test the

hypothesis that there is no real difference between the groups being compared (no effect of the experimental procedure)—that the difference observed is no greater than could be expected on the basis of "chance" alone. The validity of this hypothesis is measured by the probability, P; the smaller is P, the less the probability that the procedure was without effect. Obviously, the level at which this probability will be considered to imply significance is arbitrary, but most workers accept the conclusion of statistical significance if (a) P is less than 5 per cent, (b) the experiment proceeded "satisfactorily" with respect to the controlled and measured aspects, (c) all uncontrolled factors may be considered to have affected the groups comparably, and (d) the resulting conclusions are rational in the light of general experience and concepts.

In the present investigations, one may evaluate the effects of the experimental procedure by considering (a) the incidence of tumors—*i.e.* the percentages of mice developing tumors (by a specific time or by the end of the experiment), and (b) the time of appearance of tumors (induction time, latent period, etc.) as measured from some arbitrarily selected moment, such as that of the first application of carcinogen.

The statistical significance of the difference in *incidence of tumors*, may be measured in any of several ways, for example by the chi-square test (4). Employing these tests, the incidence of tumors in A5 was significantly less than in A0, ($\chi^2 = 18.9$, P less than 0.1 per cent) while the other pairs, A60 v A65, S60 v S61, or S10 v S11 did not differ significantly.

In order to determine statistical significance of the differences between the several pairs in *times of appearance* of skin tumors the t-test was employed (4). In this test the significance of the difference between "times of appearance" is determined from the following ratio:

$$t = \frac{M_1 - M_2}{S_d}$$

where M_1 and M_2 are the "mean" times of appearance of tumors in the two groups being compared, and S_d is the "standard error" of the difference; S_d is proportional to the variances (or "scatter") of the times of appearance of tumors in the two groups. Greater values of "t" are associated with decreased probabilities (P) that the difference could be due to "chance." Obviously then, if the standard error of the difference decreases, as the variances of the two groups decrease, there will be a tendency toward an increased value of "t" and to increased "significance" of the difference.

Bryan and Shimkin (2) have shown in studies on sarcoma-formation that, within certain limits,

increased dosage of carcinogen effects a reduction in both the mean and the variance of the times of appearance of tumors. Their data indicate that, as a consequence of this association between dosage and variance, increased dosage of carcinogen leads, for groups of equal size, to increased "reliability" of the data on times of appearance. With reference to the experiments reported here, the action of the increased dosage of carcinogen caused not only a definite decrease in the mean time of appearance of tumors but also a considerably greater decrease in the variances (A0 v A60, A5 v A65 etc.). In consequence, while the actual *magnitude* of the difference between the groups being compared was decreased by the greater carcinogen dosage (*e.g.* compare the difference between the mean times of appearance of S10 and S11 with the difference between S60 and S61) the standard error of the difference was reduced to a much greater extent. As a consequence, the values of "t" are greater (and P is smaller) for the comparisons between the groups given the greater dosages; and in these groups there was much less possibility that the differences were due to "chance."

Those familiar with the limitations of the t-test may be interested in the following considerations. One condition of the t-test is that the distributions of the times of appearance of tumors in each group do not deviate greatly from the "normal" distribution. In the present experiments none of the groups revealed statistically significant deviations from the normal distribution. Secondly, the derivation of the t-test requires that the variances of the two groups being compared should not differ significantly if the test is to afford accurate measure of the significance of the differences between the *means*. However, regardless of differences between variances, the test does measure the significance of the difference between the *distribution* of times of appearance. For example, if a value of "t" corresponds to a significantly low P, it seems relatively unimportant from a practical viewpoint whether the treatment produced only a change in the means, or in the variance also; the point is, the experimental procedure has produced a difference of statistically significant order in the times of appearance. In applying the test to the data given in Tables I and II, it was recognized that the variance of S60 was significantly greater than that of S61, and that of A65 greater than that of A60. A device commonly used to "normalize" distribution and to minimize differences in variance is the transformation of "time" to some other function such as "logarithm of time." This transformation, applied to the present data, gave some-

what better approximation to the normal distribution, and better agreement between the variances of the groups compared. The results of the logarithm transformation are given in Table III.

Regardless of the basis of comparison (time or logarithm of time) it is obvious that despite application of "large" dosages of carcinogen, the delaying effect of caloric restriction and the accelerating effect of a fat-enriched diet on the time of appearance of tumors are demonstrable. More-

contrast, the mice of the groups (A60, S60, S61, but not A65) given the larger amount of carcinogen developed 2 to 5 papillomas in the treated area; these often became confluent. As the lesions became carcinomatous differences between the low and high dosage were still apparent. The single discrete papillomas in those given the smaller dosage revealed a firm "pearly" periphery as the base, and the palisade structure of the papilloma was replaced by a horny, necrotic, or hemorrhagic

TABLE III: RESULTS OF LOGARITHMIC TRANSFORMATION OF THE DATA ON TIMES OF APPEARANCE OF SKIN TUMORS

Logarithm of the time of appearance of skin tumors (log. weeks)

Dosage of carcinogen	Group	Mean	Variance	Difference between means	Significance of difference	
					t	P
Moderate	A0: *Ad libitum*	1.403	0.0445			less than 5%
	A5: Calorie-restricted	1.553	0.0280	0.150 ± 0.063	2.3	
Large	A60: *Ad libitum*	1.203	0.00801			less than 0.1%
	A65: Calorie-restricted	1.307	0.00895	0.104 ± 0.019	5.4	
Moderate	S10: Low fat	1.470	0.0285			less than 5%
	S11: Fat-enriched	1.370	0.0558	0.100 ± 0.048	2.1	
Large	S60: Low fat	1.211	0.00823			less than 0.1%
	S61: Fat-enriched	1.133	0.00642	0.078 ± 0.018	4.3	

over, it is apparent that employment of the larger dosage increased the "statistical significance" of these differences. However, since there is probably a minimal time before which tumors may not arise despite increased dosages of carcinogen (2), it is possible that extremely large dosages might even obliterate differences in the times of appearance.

General.—In reference to the effect of dosage of carcinogen on the incidence of tumors two additional aspects deserve mention. In the experiment concerned with caloric restriction, the larger dosage of carcinogen practically eliminated the difference in incidence obtained with the smaller dosage. The data were presented without any distinction as to whether the tumors were papillomas or carcinomas, although the exact types of tumors and their progress were observed and noted in the records of the experiment. These reveal that somewhat more of the mice of the A60 group developed carcinomas than those of the A65 group.

The character and development of the tumors formed in the various groups followed, in general, a consistent pattern. In the lower dosage groups (A0, A5, S10, S11) single papillomas formed, and these were clearly demarcated from the rest of the skin to which the carcinogen had been applied. In

crust. On the other hand, the papillomas of mice given larger dosage of carcinogen were converted into carcinomas with a broader base, on the surface of which the papillomas persisted for a longer time. By the end of the experiment the differences between the groups treated with the moderate and large amounts of carcinogen were less striking, but still apparent. The inhibiting effect of caloric restriction on tumor formation was reflected in the fact that the mice of the A65 group (higher dosage, calorie-restricted) presented tumor characteristics more like those of the mice on lower dosage, rather than those of the mice of the other higher dosage groups, A60, S60, S61.

That the relative effect of the dietary procedure may be independent of the dosage of carcinogen is shown by a comparison of the times of appearance of tumors in the groups (Table IV). At both dosages of carcinogen the calorie-restricted diet prolonged the mean time of appearance by about 30 per cent; the high fat diet shortened the mean time of appearance by about 16 per cent.

For various types of tumors it has been shown that with increasing carcinogenic stimulation the incidence of tumors increases until nearly all of the treated animals develop tumors. In general, the

increasing dosage also shortens the average time of appearance (induction time) of the tumors. The correlation, between incidence and mean time of appearance of tumors, is evident not only from investigations in which the carcinogenic stimulus was the sole variant but also from studies in which the carcinogenic stimulus was constant, the variant being an experimental procedure. Loeb (7), in reference to spontaneous mammary carcinoma in different strains of mice, observed "that between

TABLE IV: RATIOS OF MEAN TIMES OF APPEARANCE OF SKIN TUMORS

Ratio	Dosage of carcinogen	
	Moderate	Large
of Calorie-restricted group to *Ad libitum* control group	1.34	1.27
of Fat-enriched group to Low fat control group	0.85	0.83

the frequency of cancer and the average age at which it appears a quantitative relation existed in the sense that, on the whole, the greater the frequency of cancer the earlier the appearance." Similarly, with regard to the many investigations performed in this laboratory on the effect of dietary changes on the formation of spontaneous and induced tumors, we have noted that the tumors in the group (control or experimental) in which the greater number occurred usually had a shorter mean time of appearance. There is a limit to the relationship, however, in that no more than 100 per cent tumors can be formed, and there is probably a minimal time before which tumors do not appear despite further increases in dose of carcinogen.

Loeb (7), as early as 1924, was of the opinion that if a stimulant was strong enough it could mask or overwhelm any inherited resistance so that all of the hosts develop cancer. Similarly, Watson and Mellanby (11) "anticipated that any resistance induced by a particular experimental treatment would be masked if the conditions for inducing the tumors were too severe." Accordingly, in experiments designed to test the modifying effect of diet or treatment of the skin on the formation of tumors, these authors utilized technics to avoid large dosages of carcinogen (tar) such as fewer paintings or applying the tar with the tip of the brush. Since then other investigators (1, 3, 10) have expressed the view that factors which influence tumor formation might have been overshadowed, obscured, or masked when large dosages

of carcinogen were employed. Others have attributed failure to reveal effects of experimental procedures to the use of large dosages of carcinogen.

To our knowledge, the first experimental support of this concept was presented in the work of Shear and his co-workers (3, 8) on the promoting effect of a basic fraction of creosote oil. Leiter and Shear (6) have reported a large number of investigations concerned, in part, with the effect of sex on the production of subcutaneous tumors in mice. They found that the percentage of tumors was greater in the males, (although not invariably) and that the ratio (incidence in males: incidence in females) decreased with increasing level of tumor production.

These considerations are of interest in light of the results reported in this communication. In conformity with these findings, the high dosage of carcinogen tended to obliterate the differences between the control and experimental groups in incidence of tumors. On the other hand, differences between times of appearance of the tumors (induction time) were actually emphasized because of increased statistical validity. The high dosage of carcinogen masked the effect of two different experimental procedures on the incidence, but made more evident the effect on the rate of formation of the tumors. This suggests that employment of two criteria—incidence and induction time—or more, might yield better evaluation of the effect of an experimental procedure. In some instances the utilization of large dosages of carcinogen might actually be advantageous, particularly in demonstrating the effects of an experimental treatment on the time of appearance of tumors.

SUMMARY

There is a prevailing view that large dosages of carcinogen may obliterate the effect of an experimental procedure on the induction of tumors. This is reasonable since the incidence of tumors increases with increasing amounts of carcinogen, finally approaching the maximum of 100 per cent. Since, in general, increasing incidence of tumors is associated with the shortening of the mean time of appearance of these tumors, it seemed worthwhile to inquire into the effect of large dosages of carcinogen on the latter criterion of tumor formation.

The inhibiting effect of caloric restriction and the enhancing effect of a fat-enriched diet on the formation of induced skin tumors was investigated at two levels of carcinogen dosage, moderate and high. The results showed that large amounts of carcinogen tended to mask the action of the experimental procedures in regard to incidence of tumors. On the other hand, the effect on time of

appearance (induction time) of the tumors was actually emphasized through increased statistical validity.

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The Influence of Mammalian Environments on the Tissue Specificities of the Rous Chicken Sarcoma Virus*

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Since the Rous chicken sarcoma virus undergoes variation in its tissue specificities following growth of the tumor in the anterior chamber of the guinea pig eye (6), it was considered of interest to determine whether other mammalian hosts have a similar influence on this agent. The Rous tumor, therefore, has been transplanted into the anterior chamber of the mouse eye in an effort to see first if growth of the tissue would take place, and second if the virus would undergo variation. The present communication gives in detail the results of these experiments, and compares them with those already reported for chicks inoculated with the guinea pig passage tumor.

MATERIALS AND METHODS

Avian hosts.—In all cases, chicks obtained from a local commercial hatchery were of the Plymouth Rock breed. These birds varied in age from 1 to 60 days at the time of injection. The different strains of Rous tumor were maintained in chicks after a single rodent passage, tissue specificities of the virus being observed in the avian hosts. Only those birds presenting one or more of the protean lesions of the Rous virus infection are included in the present data. All inoculated individuals, both experimental and controls, were kept together in the same brooder.

Mammalian hosts.—The guinea pigs used were obtained from the random bred stocks of local dealers, and varied in age from 6 to 8 months (6). On the other hand, the mice utilized for intraocular inoculations of the Rous tumor were young adults of the "A" inbred strain. A few individuals of the C3H stock also were inoculated, but observations were more difficult in the pigmented eye of the agouti than in the pink eye of the albino, and use of the former strain was abandoned. All of the data to be considered here deal with observations on tumors passed through "A" strain mice.

Tumor material.—The strain of Rous tumor used in the guinea pig experiments was obtained from Dr. F. Duran-Reynals. This sarcoma has been under study by him for the past 7 years. Mouse inoculations were carried out with a strain of the Rous sarcoma which was obtained in 1944 from Dr. Albert Claude of the Rockefeller Institute. Both tumor strains yield virulent virus which causes pronounced lesions of all gradations ranging from those predominantly hemorrhagic to those entirely neoplastic.

Since the differences in the incidence of involvement of the various chicken tissues by the two stock viruses did not vary beyond the limits of random sampling, the observations within these two groups of birds were pooled and used as controls.

Methods.—The technic employed for the transplantation of tumor tissue into the mouse eye is essentially that used for the guinea pig inoculation (6). Sarcomas for transfer were obtained from freshly killed birds. These growths had developed in the breast following an intramuscular injection of tumor filtrate (Berkefeld "N") 10 days to 2 weeks previously. For guinea pig inoculation the tumor tissue was divided into small pieces approximately 2 to 3 cu. mm. in size, and placed into a No. 2 trocar. The material for mouse injection was cut into considerably smaller portions since these were made to fit into a 22-gauge spinal needle (5). The anterior chamber of the rodent eye was opened by plunging the point of a Bard-Parker No. 11 blade through the cornea at the sclerocorneal junction. The fragment of tumor to be transplanted was inserted into the eye by means of the trocar or needle and forced to the lower portion of the chamber by means of the stilet plus slight pressure on the corneal surface.

The guinea pigs were killed 10 or 11 days after inoculation and the growths were transferred to the breast muscle of chicks by means of the trocar. The mice were sacrificed at various times from the first to the 29th day after injection and the transplants were inserted into avian hosts in a manner similar to the above. From these rodent passages, then, two lines of Rous tumors were established in chicks; the one coming from the guinea pig pas-

sage is referred to in this paper as the "guinea pig passage tumor", and the other from the mouse, the "mouse passage tumor". The sarcoma strains that have never been inoculated into either rodent are referred to as "stock Rous tumor".

Following the transfer of the tumor from the rodent to the chick, subsequent inoculations were made into birds by either the intramuscular route into the breast (cell suspension diluted $\frac{1}{5}$ by weight) or a combination of the intravenous route into the external jugular and intramuscular route into the breast with Berkefeld "N" filtrates (dilution 1/20 by weight). The data here presented have been obtained from observations on birds representing from 5 to 8 serial passages of guinea pig passage tumor, and on 5 avian transfers of the mouse passage material. It should be emphasized that the observations on the guinea pig passage tumor material represent a pooling and further analysis of data already presented (6) and stem from growths obtained from the inoculation of 3 different guinea pigs. On the other hand in the present experiments 23 mice yielded tumors that grew in chicks, and the data from the mouse passage material is obtained from pooled observations of these lines over 5 serial bird passages.

EXPERIMENTAL

Properties of the guinea pig passage tumor strain.— The chicks receiving the Rous tumor directly from the rodent did not show any unusual distribution of tissue involvement. Three of the 9 birds inoculated with this material developed hemorrhagic lesions, thus indicating that the virus had persisted throughout the residence of the transplant in the rodent eye. Further, 6 chicks had a tumor localized at the inoculation site in the breast and the other 3 showed an extension of the local lesion to the liver. There was no evidence of periosteal growths in these individuals of the first avian passage. Subsequent transfer of these tumors into chicks gave evidence that the tissue specificities of the virus had changed. While many new areas of the host were involved by the virus the development of periosteal tumors was considered to be the most consistent indication of virus alteration (2). Of 121 birds inoculated with Berkefeld "N" filtrates of this tumor strain, 43 per cent subsequently possessed one or more bone lesions. Evidence suggests that this alteration in tissue specificities of the virus may not be a transient affair. Throughout 8 chicken passages the frequencies of those with and without bone tumors did not vary beyond the limits of random sam-

pling.[1] However, the total number of birds in the last 2 passages is small. The frequency of bone lesions in the sixth chicken passage is the most out of line (Table I).

TABLE I: INCIDENCE OF BONE LESIONS IN BIRDS OF SUBSEQUENT CHICKEN PASSAGES INOCULATED WITH FILTRATES OF THE GUINEA PIG STRAIN OF ROUS TUMOR

No. of birds with	No. of chicken passages							Total No. of birds	Prob- ability†
	2	3	4	5	6	7	8		
Bone lesions	9	13	9	10	5	4	2	52	
									0.42
No bone lesions*	8	13	11	14	16	3	4	69	

*Those birds not showing bone lesions all had other manifestations of Rous virus infection.
†The probability that these two distributions are samples from the same population. Probabilities of 0.01 or less are the only values of "P" considered significant.

Other tissues of the chick not normally involved were also invaded by the altered agent, but not consistently enough to warrant statistical analysis. The most interesting of these was the occurrence of 2 ulcers in the mucosa of the gizzard (Fig. 1). Fig. 2 shows these lesions to be invasions and eventual replacements of the mucosa by the malignant fibroblasts, the tumor probably arising from the submucosal area.

Morphological evidence that the growths of the guinea pig passage strain metastasize by cell emboli in the blood stream may be seen in Fig. 3. Also these growths actually invade tissue cells (Fig. 4). The histology of this rodent tumor strain was essentially the same as that found in the stock Rous growths no matter whether the transplant was in the rodent or chick. The behavior of the chicken sarcoma in the guinea pig eye has been described (6).

Growth of the Rous chicken sarcoma in the mouse eye.—Of the 31 mice inoculated intraocularly with this sarcoma, 23 yielded growths, resulting in tumors on injection into chicks. The criteria for growth of the transplant in the rodent were: (a) the increase in size of the transplanted tissue, (b) the presence of mitotic figures in the tumor, and (c) the ability of the transplant to produce growths in chicks. The second criterion was found not to be too reliable since mitoses were frequently difficult to find yet the other two criteria were easily fulfilled. Due to the size of the eye it was difficult to determine the onset of vascularization. The transplanted tissues were allowed to remain in the mouse eye for periods varying from 1 to 29 days.

[1] In the present study, probabilities as determined by chi-square tests of 0.01 or less are the only values of "P" considered significant.

During this time growth was manifest by an increase in size, frequently associated with a bulging of the cornea, and in some cases by a herniation of the growth through the outer surface of the eye (Figs. 6 and 9). In Fig. 9 it may be seen that the tumor tissue has invaded between the corneal layers with the result that it is almost completely surrounded by stratified squamous epithelium. Fig. 10 gives a higher magnification of this transplant and shows the "fingers" of epithelial cells

mouse eye, 43 developed tumors; 31 of these were localized in the region into which the eye transplant was placed. Eight of the 43 individuals possessed tumors also in the lungs, and 3 others showed extension of the local growth to the liver. Nine birds receiving the sarcoma directly from the rodent possessed hemorrhagic lesions of varying intensities, whereas 2 chicks developed periosteal growths following inoculation of the anterior chamber transplants. There was no correlation

TABLE II: The Probabilities That Weighted Mean Ages of the Birds Being Compared do not Vary Beyond the Limits of Random Sampling

Chicks inoculated with tumor, Strain	Inoculum	Birds with lesions	No. of birds inoculated	Weighted mean age		Standard error	Probability*
Stock Rous			23	14.87	±	2.05	—> < 0.01
Guinea pig	Filtrates	Bone	52	8.73	±	0.69	—> < 0.01
Mouse			41	9.15	±	0.68	—> > 0.60
Stock Rous			78	11.83	±	0.94	—> < 0.01
Guinea pig	Filtrates	Hemorrhagic	97	7.76	±	0.41	—> < 0.01
Mouse			148	7.41	±	0.40	—> > 0.50
Stock Rous			111	16.57	±	1.21	—> < 0.01
Guinea pig	Cell suspension	Hemorrhagic	84	11.93	±	0.68	—> < 0.01
Mouse			33	5.09	±	0.60	—> < 0.01
Stock Rous			105	11.90	±	0.84	—> < 0.01
Guinea pig	Filtrates	All manifestations of virus infection	121	7.65	±	0.39	—> < 0.01
Mouse			237	7.34	±	0.31	—> > 0.50
Stock Rous			445	15.37	±	0.52	—> < 0.01
Guinea pig	Cell suspension	All manifestations of virus infection	153	11.22	±	0.66	—> < 0.01
Mouse			179	6.03	±	0.28	—> < 0.01

*The probabilities were determined by means of the "t" test.

invading the tumor. After about 15 days' residence in the rodent eye the size of the tumor decreases and eventually no trace of the growth remains. A corneal scar may persist, however. Successful transfer of growths from mice to chicks has not been achieved after the 15th day. Serial passage of the Rous sarcoma in the mouse has not been possible as yet beyond the second rodent transfer.

Histologically the tumor in the mouse possessed no features distinguishing it from that seen in stock Rous tumor material (Figs. 5, 7, and 8). Fig. 11 represents the architecture of the tumors of the mouse material in chicks. It may be seen that there is little change from the original growths. Some of these sarcomas contain considerable mucin.

Properties of the mouse passage tumor in chicks.— Of the 50 chicks that received transplants from the

between the length of time the sarcoma remained in the rodent and the type of lesions present in the chick.

Probably the most significant point to be noted in the behavior of the mouse passage strain in chicks is that the incidence of bone lesions among the birds inoculated with filtrates is low (Table III). Fig. 12 illustrates the histology of these lesions and it may be seen that there is no essential difference between this and the periosteal growths of the guinea pig passage tumor (6). On one occasion, however, there was evidence of a marked increase in cortex bone associated with the periosteal tumor (Fig. 13). Fig. 14 shows a higher magnification of this tissue and it can be seen that some of the osteoid tissue is undergoing degeneration.

The incidence of hemorrhagic disease in these
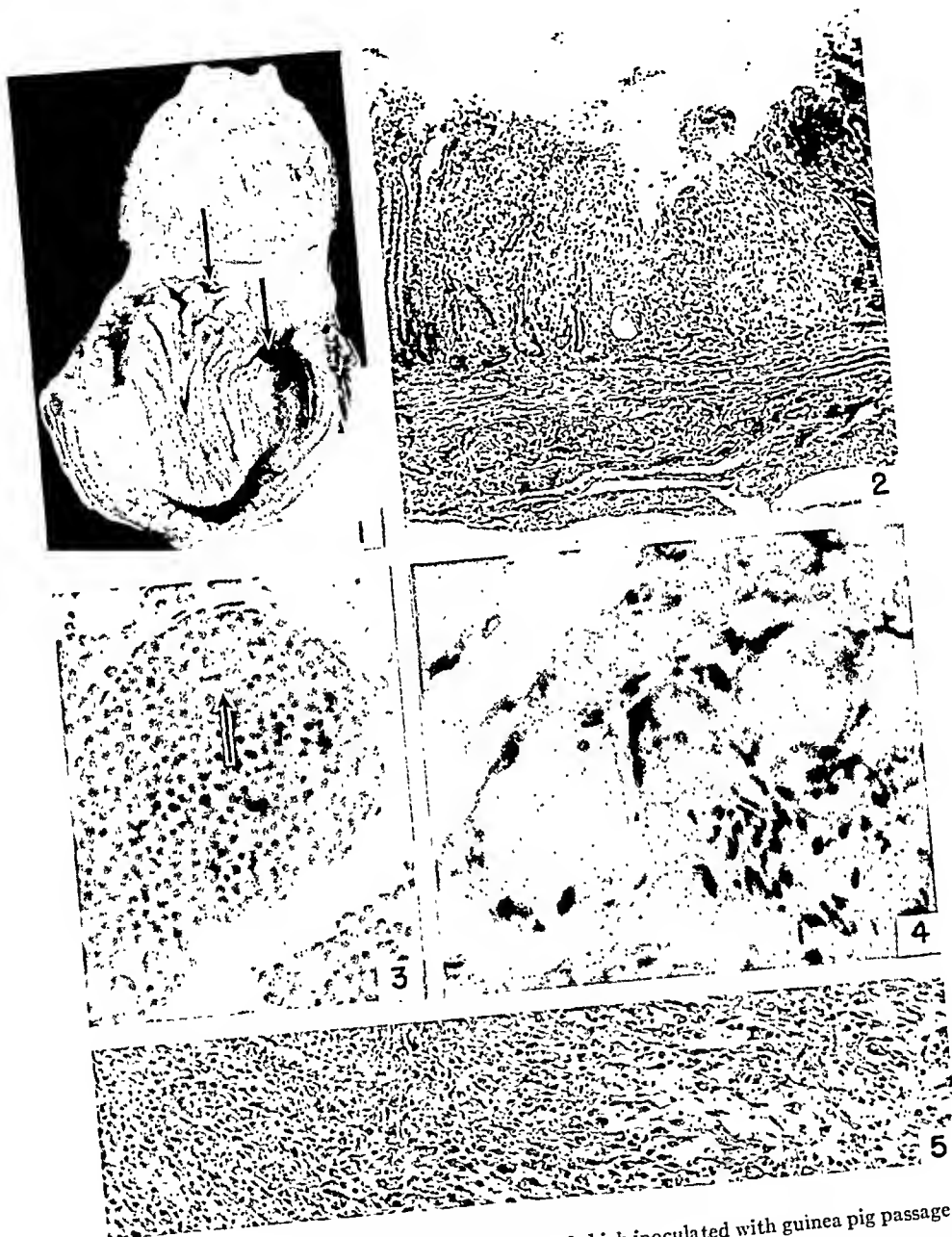
FIG. 1.—Ulceration of gizzard mucosa due to infiltration of cells of Rous sarcoma of guinea pig passage strain.

FIG. 2.—Invasion and replacement of cells of gizzard mucosa by sarcoma cells. Mag. × 100.

FIG. 3.—Embolus of sarcoma cells in pulmonary vessel of chick inoculated with guinea pig passage material. Mag. × 800.

FIG. 4.—Invasion of muscle cell by malignant fibroblasts of guinea pig passage tumor. Mag. × 800.

FIG. 5.—Histology of Rous chicken sarcoma used as control material. Mag. × 160.
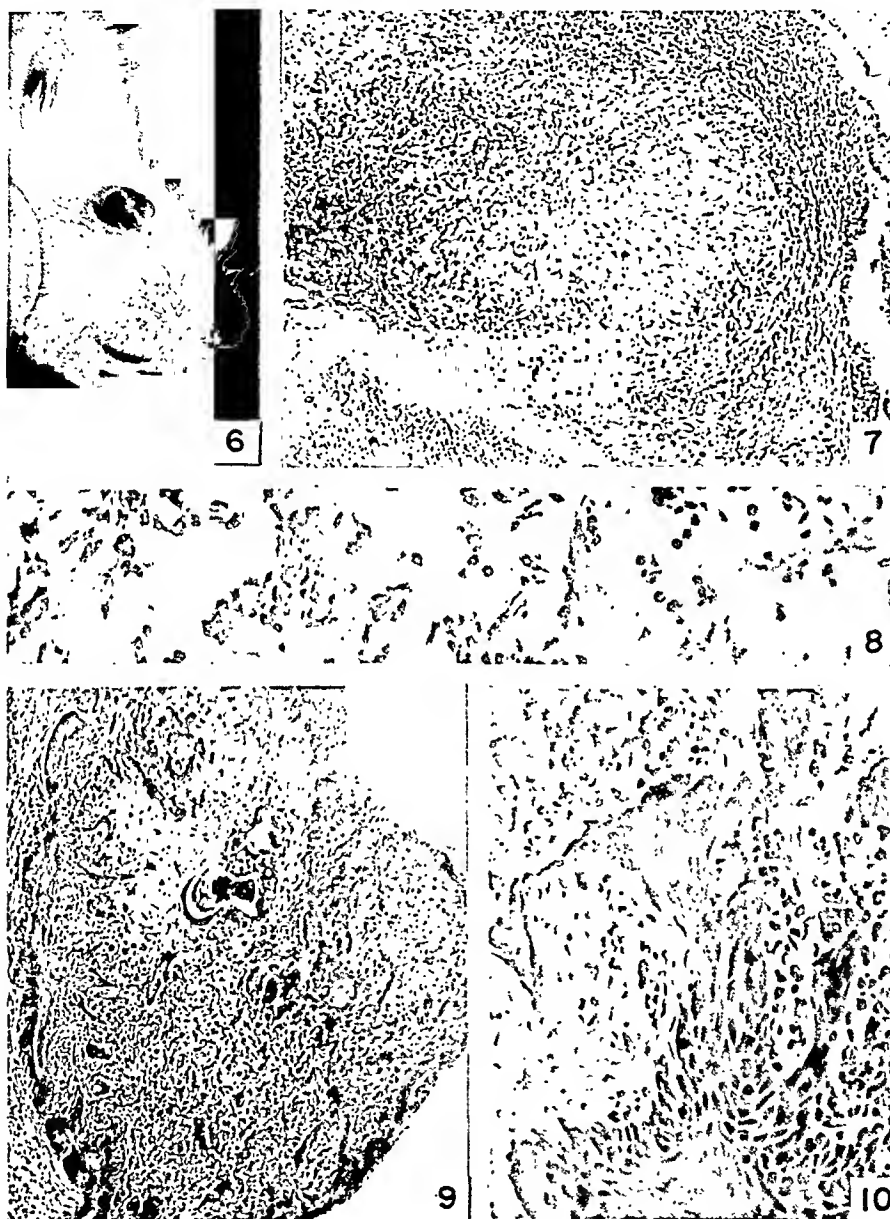
FIG. 6.—The Rous sarcoma growing in anterior chamber of mouse eye.

FIG. 7.—Histological architecture of Rous sarcoma growing in anterior chamber of the mouse eye. Mag. × 85.

FIG. 8.—Tissue seen in Fig. 7. Mag. × 600.

FIG. 9.—Herniation of sarcoma in mouse eye through cornea. The tumor has invaded between layers of cornea and has ulcerated through outside surface. Note "fingers" of stratified squamous epithelium entering the tumor. Mag. × 85.

FIG. 10.—Section seen in Fig. 9. Mag. × 365.

birds inoculated with mouse passage tumor may be seen in Table V, while the distribution of the intensities of the hemorrhagic lesions is presented in Table VI. Those individuals inoculated with cell suspensions of this tumor strain possessed growths in the majority of instances at the injection site only (Table VII). This was likewise true, but not to the same degree, for those receiving filtrates of the sarcoma. On the other hand 6.7 per cent of the birds with filtrates gave no tumors at all.

arose as to whether there was a greater likelihood of multiple bone lesions occurring in birds inoculated with the guinea pig strain than in those injected with either the mouse or control tumors. Table IV shows that no matter what the origin of the inoculum may be, the bird developing one bone lesion has the same chance, within the limits of random sampling, of developing more.

Of 445 chicks inoculated with cell suspensions of the stock Rous growth only 7 developed bone tumors. Two birds in the mouse passage group

TABLE III: A COMPARISON OF THE INCIDENCES OF BONE TUMORS IN CHICKS INOCULATED RESPECTIVELY WITH FILTRATES FROM THE THREE TUMOR VIRUS STRAINS

Filtrate from*	Birds with bone lesions		Birds without bone lesions		Total no. of birds inoculated	Probability†
	No.	%	No.	%		
Guinea pig passage tumor	52	43.0	69	57.0	121	—> <0.01
Mouse passage tumor	41	17.3	196	82.7	237	—> <0.01
Stock Rous tumor	23	21.9	82	78.1	105	—> 0.33

*See text for explanation of terms.
†The probability that the two groups being contrasted are random samples from the same population.

A comparison between the properties of the 3 strains of the Rous sarcoma virus.—Since the types of clinico-pathological manifestations of the Rous virus infection may depend upon the age of the host, the weighted mean age of the birds in each of the groups to be compared appears in Table II. It can be seen that the ages of the birds injected with the stock Rous strain were significantly different from those inoculated with the other two. However in only two instances did the mean ages of the chicks inoculated with the guinea pig and mouse strains vary beyond the limits of random sampling and these received cell suspensions.

A study of the effects of the two mammalian environments upon the properties of the Rous tumor virus was achieved by a detailed comparison of the frequencies of the lesions described above. Table III shows that significantly more bone lesions occurred in chicks inoculated with filtrates of the guinea pig strain of tumor than in chicks injected with either of the other two virus strains. On the other hand the mouse passage material did not differ significantly from the stock Rous control in this regard. The incidence of 21.9 per cent periosteal tumors in the stock Rous-infected birds suggests that the virus may undergo spontaneous alteration in its tissue specificities.

Occasionally all 3 of the virus strains produced more than one bone lesion in chicks injected. In fact, in 1 bird as many as 7 bone tumors were present. The locations included not only the long bones but also the pelvis and skull. The question

and 6 in the guinea pig passage series possessed similar lesions, 179 and 153 individuals being included in the respective groups.

Another property of the 3 virus strains which lends itself to comparison is the relative ability of the infectious agents to produce hemorrhagic disease in their respective hosts. Table V represents the frequencies, with their corresponding percentages, of hemorrhagic lesions which appeared in individuals of the three bird populations. It can be seen that in general there is a greater tendency for the guinea pig passage virus to produce hemorrhagic lesions than either the mouse or stock agents. When filtrates of the growths were used, the number of birds which showed these lesions was slightly less in the group receiving mouse material than in the control population. Definitely more chicks showed hemorrhages following injection of guinea pig passage tumor than did those getting mouse material. There was essentially no difference between the guinea pig and control viruses when

TABLE IV: THE INCIDENCE OF MULTIPLE BONE LESIONS OCCURRING IN CHICKS INOCULATED WITH FILTRATES OF THE VARIOUS STRAINS OF THE ROUS SARCOMA

Filtrate from	No. of birds with 1 bone lesion	No. of birds with more than 1 bone lesion	Total No. of birds with bone lesions	Probability
Guinea pig passage tumor	32	20	52	—> 0.80
Mouse passage tumor	19	22	41	—> 0.25
Stock Rous tumor	11	12	23	—> 0.68
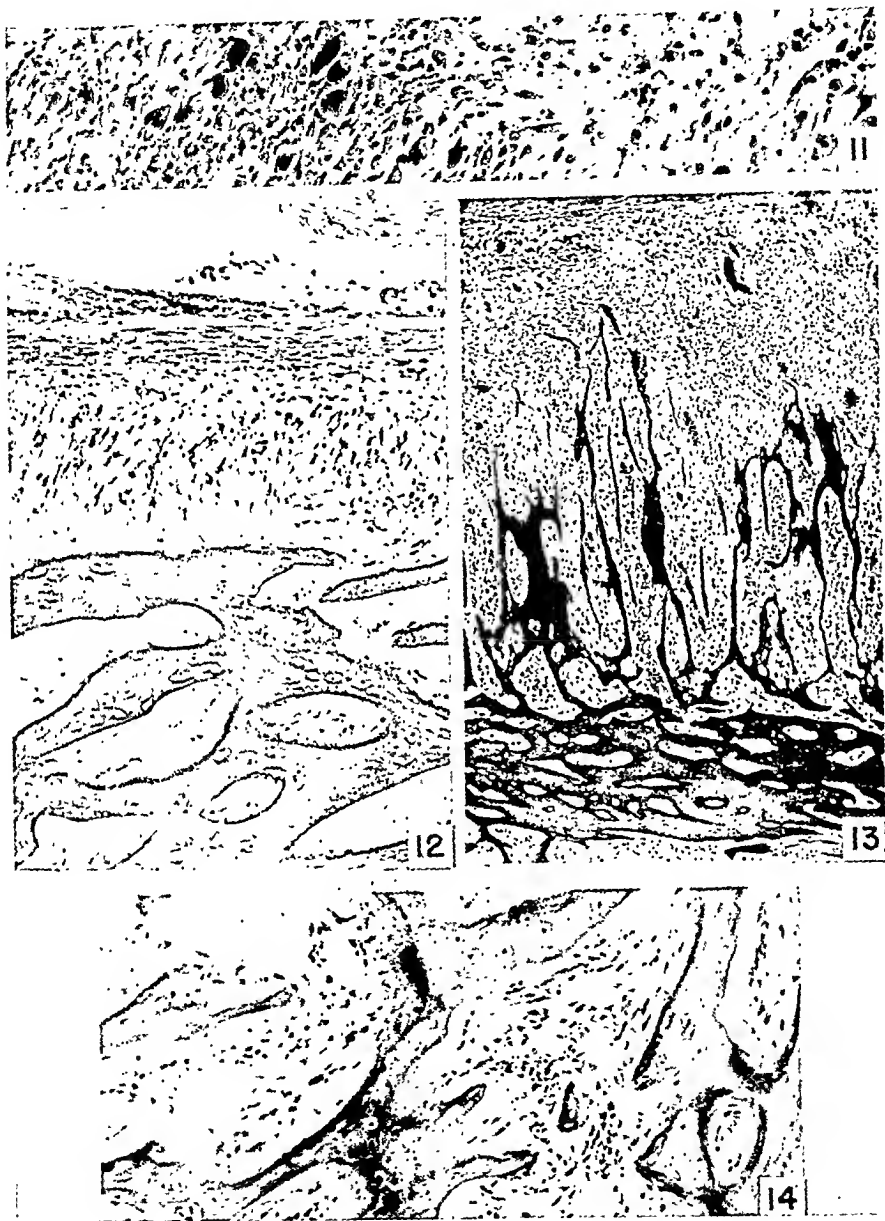
FIG. 11.—Sarcoma in chick inoculated with transplant from mouse eye following residence in rodent of 15 days. Mag. × 240.

FIG. 12.—Periosteal tumor in chick inoculated with mouse passage tumor virus. Histologically the periosteal growths were similar regardless from which of the three strains the virus was taken. Mag. × 250.

FIG. 13.—Bone tumor in which new cortex has been laid down. This is now undergoing regression. The bird was injected with mouse passage tumor virus. Mag. × 85.

FIG. 14.—Tumor seen in Fig. 13. Mag. × 250.

filtrates were used. With cell suspensions, however, the guinea pig passage virus produced definitely more hemorrhagic lesions than either of the other two strains.

What the effect of age of the birds injected may have on these results is not clear, but at most it is probably a minor factor in the range we are working. This is borne out by 3 points: one, the fact that birds injected with stock and mouse strains varied in age but not in the frequency of bone and hemorrhagic lesions; two, birds with and without

organ or several large blebs only on 1 organ. Finally those chicks in class 3 show large lesions in abundance distributed over many organs. From Table VI it can be seen that the frequencies among the chicks inoculated with filtrates of the respective tumor strains show little or no difference in classes 1, 2, and 3 for the three groups. However, in those chicks injected with the cell suspensions, the guinea pig passage material has a greater tendency to produce the most severe manifestations, in contrast to the other two strains.

TABLE V: A COMPARISON OF THE INCIDENCE OF HEMORRHAGIC LESIONS IN CHICKS INOCULATED WITH VARIOUS STRAINS OF THE ROUS SARCOMA

Filtrate from	Birds with hemorrhagic lesions		Birds without hemorrhagic lesions		Total no. of birds inoculated	Probability
	No.	%	No.	%		
Guinea pig passage tumor	97	80.0	24	20.0	121	—> <0.01
Mouse passage tumor	148	62.5	89	37.5	237	—> 0.41
Stock Rous tumor	78	74.3	27	25.7	105	—> 0.04
Cell suspension from						
Guinea pig passage tumor	84	55.0	69	45.0	153	—> <0.01
Mouse passage tumor	33	18.4	146	81.6	179	—> <0.01
Stock Rous tumor	111	24.9	334	75.1	445	—> 0.13

hemorrhagic lesions within a specific inoculated group were essentially the same age; and three, the variations between the frequencies of the lesions obtained were significant even though the birds receiving guinea pig and mouse strain viruses did not differ significantly in age.

Attempts have been made to quantitate the intensity of these lesions. The extent of hemorrhagic disease has been arbitrarily graded according to three classes: 1, 2, and 3. Class 1 includes those birds showing a few small hemorrhagic blebs scattered over one organ, or 1 large bleb on a single organ. Class 2 represents individuals possessing scattered small lesions over more than 1

A comparison between virus strains of the various locations of tumors developing after either intravenous plus intramuscular inoculation of filtrates on the one hand, or intramuscular injection of cell suspensions on the other, may be seen in Table VII. The incidence of individuals showing only local lesions at the site of inoculation did not vary significantly among the three groups of birds when filtrates were administered. However, in the case of cell suspensions, each group differed from one another beyond the limits of random sampling, the guinea pig strain eliciting less local tumors than the stock virus, and the mouse strain showing the greatest tendency for such localiza-

TABLE VI: THE DISTRIBUTION IN PER CENT OF THE INTENSITY OF HEMORRHAGIC LESIONS AMONG CHICKS INOCULATED WITH VARIOUS STRAINS OF THE ROOS SARCOMA

Filtrate from	Intensity of hemorrhagic lesions*			Total birds inoculated	Probability
	1	2	3		
Guinea pig passage tumor	10.3	24.7	65.0	97	—> 0.04
Mouse passage tumor	24.4	18.9	56.7	148	—> 0.02
Stock Rous tumor	25.6	25.6	48.8	78	—> 0.46
Cell suspension from					
Guinea pig passage tumor	19.0	19.0	62.0	84	—> <0.01
Mouse passage tumor	45.6	27.2	27.2	33	—> <0.01
Stock Rous tumor	43.2	28.8	28.0	111	—> 0.76

*See text for explanation.

tion. Those birds possessing, in addition to local tumors, growths in the lungs and those birds with extension of their local sarcomas to the liver were not compared statistically since it was felt that these manifestations of infection were too dependent upon the technic of injection to represent true properties of the infectious agents. In spite of the incidence of 11.4 per cent of chicks showing the general spread of tumors throughout the body in the mouse passage strain, this value did not vary significantly from the frequencies of similar lesions

with the control tumor virus. The frequency of these lesions in the birds inoculated with cell suspensions was considerably less in all the three bird groups than when filtrates of the tumors were used.

Since bone lesions indistinguishably histologically from those seen in the chicks injected with rodent passage tumors were present in the control birds, it would suggest that some alteration in tissue specificities of the virus occurs naturally and the guinea pig passage merely speeds up this process while mouse passage does not alter it at all.

TABLE VII: THE FREQUENCY OF VARIOUS TYPES OF TUMOR RESPONSES IN BIRDS INOCULATED WITH CELL SUSPENSIONS OR FILTRATES OF VARIOUS ROUS TUMOR STRAINS

Percentage of birds in the 3 groups with tumors

Birds with*	Stock Rous tumor		Guinea pig tumor		Mouse tumor	
	Cell susp	Filt.	Cell susp.	Filt.	Cell susp.	Filt.
Local tumors	72.4	62.8	48.4	62.8	87.8	74.7
Local tumors plus lung growths	15.0	27.6	32.0	8.4	7.3	4.2
Local tumors plus liver growths	11.9	0.9	17.6	8.4	3.3	3.0
Generalized tumors	0.7	6.8	2.0	5.7	1.1	11.4
No tumors at all†	0.0	1.9	0.0	14.7	0.5	6.7
Total number of birds observed	445	105	153	121	179	237

*See text for explanation.
†Those birds not showing tumors had other manifestations of Rous virus infection.

in the other two populations. One significant observation made, recorded in Table VII, is that the Rous sarcoma virus apparently lost some of its ability to produce tumors in chicks by virtue of its sojourn in the guinea pig. Approximately 14 per cent of the individuals receiving the guinea pig passage agent failed to develop growths although other manifestations of the virus infection were present. The percentage of birds injected with the mouse passage filtrate not developing tumors, represented a frequency that was not significantly different from that observed in the control chicks.

DISCUSSION

It has been possible to grow the Rous chicken sarcoma in the anterior chamber of the guinea pig eye as well as in the eye of the mouse. The subsequent inoculation of the filtrates and cell suspensions of these two strains of sarcoma into chicks has yielded information suggesting that the causative virus that has passed through the guinea pig has become altered in its tissue specificities, as seen by the increase in the incidence of periosteal lesions. On the other hand, the agent that had sojourned in the mouse eye did not show an incidence of bone lesions in avian hosts differing significantly from that found in the birds injected

Regardless of the tumor strain from which the filtrates are obtained, the probability of inoculated chicks developing more than one bone lesion is not significantly different in the three host groups. This further suggests that the alteration in the guinea pig virus may be just an accentuation of a natural phenomenon.

Guinea pig passage tumor virus had a greater tendency to produce hemorrhagic disease in chicks than had either of the other two virus strains. This was particularly clear when birds were inoculated with cell suspensions. Moreover these individuals of the guinea pig passage strain possessed more severe hemorrhagic lesions than the members of the other two groups.

Although the incidence of solitary lesions at the site of inoculation among the birds of the three populations was not significantly different when filtrates were used, there was a definite tendency with cell suspensions for the guinea pig virus to produce less localization than the mouse passage agent. In fact there was a suggestion of a greater spread of lesions in the control infections than in the birds injected with mouse passage strain. Although the three strains of viruses differed regarding the incidence of tumors at the site of inoculation, there was no greater tendency for any one strain to give generalized tumors throughout the

body. The guinea pig as well as the stock Rous virus may have a greater tendency to produce tumors in the lungs, and growths in the liver in addition to their local lesions than the mouse strain virus, albeit these data were not analyzed statistically for reasons stated above. Finally, definitely fewer birds developed tumors on inoculation with the filtrate of the guinea pig passage virus, yet these chicks possessed other manifestations of the Rous infection. Study of this non-tumor group showed that age was not a factor. It appears, therefore, that by virtue of the guinea pig passage the Rous virus has lost some of its ability to produce tumors in chicks when filtrates are used. This might suggest an alteration in the species specificities of the Rous virus.

Since Duran-Reynals (1) has shown that hemorrhagic lesions in the chick are the result of an interplay of virus virulence and host resistance and that this host resistance may be closely associated with age, the present results suggest that the potency of the Rous virus has been increased by guinea pig passage. Whether this alteration in potency is a qualitative or quantitative change can not be said at present. Furthermore, the Rous agent was so altered in its tissue specificities by its sojourn in the guinea pig that it produced bone tumors in greater abundance. On the other hand, the mouse passage virus possessed properties, concerning potentialties for the production of hemorrhagic and bone lesions, which were more closely allied with those observed for the stock Rous agent. On working with the mouse virus strain one gains the impression that actually the potency of this virus is less than that of the control. The only statistical evidence that might support this theory is the observation that cell suspensions of the mouse virus produce more solitary tumors in birds at the site of injection than either of the other two strains.

The age of the host, within the range of 1 to 60 days, is probably not an important factor in the manifestation of the various lesions produced by the virus strains. While the mean ages of the birds in the various groups vary significantly from each other, those showing wide discrepancy of age gave no significant differences in the incidence of bone and hemorrhagic lesions. On the other hand, the birds receiving guinea pig and mouse strain virus showed significantly different frequencies of these lesions, yet these chicks did not vary in age beyond the limits of random sampling.

The behavior of the Rous sarcoma in the mouse eye is practically identical with that of the sarcoma in the anterior chamber of the guinea pig eye, with the possible exception that its period of growth is longer. Mammalian cancer in these environments has been described by Greene (4 and 5), and the activities of these growths in contrast with those of the Rous sarcoma have been discussed (6). With reference to the behavior of human tumors in the mouse eye Greene (5) has noted that great difficulty is encountered in transplanting these growths directly from man to mouse. However, following growth in the guinea pig eye the human neoplasm will grow readily in the mouse, eventually killing the animal.

SUMMARY AND CONCLUSIONS

By the inoculation of the Rous Chicken sarcoma into the anterior chamber of the eyes of mice and guinea pigs, studies have been made possible concerning the effects of these two rodent hosts upon the specificities and potencies of the respective tumor viruses. It was found that the guinea pig had a more pronounced influence upon these properties than the mouse. In the former, changes were manifested in chicks by the increase of the incidence of periosteal and hemorrhagic lesions, and by the decrease in the appearance of localized growths at the inoculation site of the filtrates. On the other hand the virus that was passed through mice was essentially similar in properties to the agent experiencing no rodent passage.

These results are interpreted as suggesting that residence of the Rous tumor in the guinea pig eye increased the potency of the virus and altered its tissue and species specificities. By contrast the passage of the sarcoma through mice had no such effect. In fact there was a possibility that the potency of the virus was actually decreased by this procedure.

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Organophilic Tendencies of Two Transplantable Tumors of the Mouse*†

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INTRODUCTION

Relationships between the transplanted tumor and its host.—A growing neoplasm that will eventually cause the death of the host may or may not develop daughter colonies, or metastases. When a mouse tumor is successfully transplanted into new hosts the same tendency either to metastasize in a certain percentage of the host mice or to remain localized is apparently shown by the tumors of later tumor tissue transplant generations. In other words some implanted tumors characteristically remain at the site of inoculation while others may not only develop at the foci of transplantation but also be transferred to other parts of the body. Certain human neoplastic growths have characteristic sites for metastases such as the bones in some cases of carcinomas of the breast and of the prostate. The tumors of mice in general may metastasize by means of tumor cell emboli that most commonly go to the lungs. Ewing states "That tissue—and tumor—cells pass through the pulmonary capillaries has frequently been demonstrated, and in certain tumors, especially the lymphosarcomas, general metastases develop in this way. Passing the lungs the embolic cells tend to lodge in organs with feeble circulation, such as liver, bone marrow, and subcutaneous tissue." (3)

This explanation is not sufficient to account for the relationships between the different host types and the transplantable tumor that will be analyzed in this paper. These data present a comparative study of two different tumors. However, the primary objective here is to analyze the host-tumor relationship where the same transplantable tumor has been observed in a variety of different genetically controlled host types. The systemic response for each host type has been studied in order to determine which internal organs show gross evidence of tumor involvement concurrent with the growth of neoplasms at the subcutaneous implantation site. It is hoped that these data will clarify the following points: (a) If a tumor metastasizes, does it involve the same organs in all types of hosts? (b) Does the percentage of metastastic growths of the same tumor vary for different types of hosts? (c) Does this frequency of metastases vary for the same organs within a host type? (d) If an organophilic tendency is associated with tumor metastases, is this dependent upon the tumor, the host, or upon a host-tumor relationship?

Spontaneous neoplasia in C57 leaden strain mice.—The C57 leaden strain mice arose by mutation from the C57 brown strain (7). The total tumor incidence has not been tabulated, but there are certain general observations concerning spontaneous tumors that can be stated here. Although several hundred careful autopsies were carried out on old leaden mice, only 1 tumor of mammary gland origin has been seen during a period of 12 years. This mammary tumor was nontransplantable and on sectioning proved to be an adenoma. Primary lung tumors have been nearly as rare as tumors of the mammary gland. We have found fibrosarcomas and leukemias somewhat more frequently, probably at about the same rate as has been recorded for the C57 black stock (6). If any one body site in the leaden mice is the most frequently involved by spontaneous tumors, it would appear to be the liver. The growths found in this organ have usually been either parenchyma cell tumors or reticuloendotheliomas (histiocytomas). A representative of each of these two tumor types from the liver was selected for the following studies by transplantation.

MATERIALS AND METHODS

Types of animals used.—Several types of mice were employed. These were strains C57 black line 6 (B), C57 brown lines *a* and *cd* (Br^a and Br^cd), C57 leaden (L), dilute brown (dba), and albino (A). First generation hybrids between several of these strains were also tested. The C57 strains are not closely related; their origin from a common hybrid ancestry is diagrammatically shown.

Tumors employed.—A liver carcinoma (C954) and a reticuloendothelioma (C198) were studied

with respect to their organophilic tendencies following transplantation. Tumor C954 was first seen in 1940 as a spontaneous tumor of the parenchyma cells of the liver. It arose in an old male of the leaden strain. This carcinoma has been carried in leaden mice for more than 60 tumor transplant generations. The other spontaneous tumor, C198, also originally involved the liver of a leaden strain mouse. It was observed in an old breeding female that was autopsied in 1936 (1).

tissue for further inoculations. After the first transfer, tumor C954 has grown subcutaneously in approximately 100 per cent of all leaden strain mice inoculated.

Method of recording data.—Complete necropsies were performed upon practically all these mice when they became moribund from advanced tumor growth. If the animals were resistant to tumor inoculations they were autopsied at the end of 6 to 9 months. Mice developing sub-
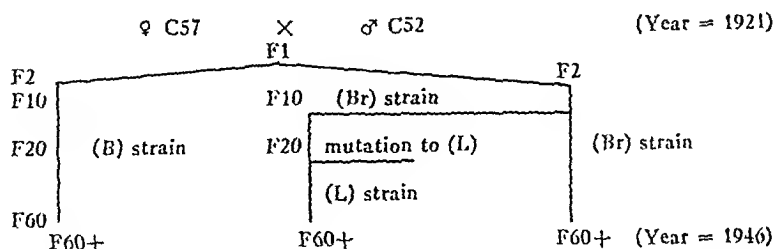
In 10 years this tumor passed through 145 tissue transplant generations in leaden strain host mice.

Method of tumor transfer.—The usual trocar method was employed and the implants were routinely made into the subcutaneous tissues in the right axillary region. The neoplastic liver tissue was the source of the tumor tissue used in the initial transfer of both spontaneous tumors C954 and C198 to other leaden strain mice. After the first transfer the source of the tissue for subsequent inoculations differed for these 2 tumors.

For more than 10 years the reticuloendothelioma C198 had been carried in the leaden strain from host to host by injecting fragments from the tumorous livers rather than from the subcutaneous tumors. This practice was necessary at first because only 10 mice of the 111 inoculated in the first 5 transfer generation developed any subcutaneous masses at the site of injection in the right axillary region. These neoplasms were but a few millimeters in diameter. Nevertheless, all the leaden strain mice injected subcutaneously, developed tumor lesions in the liver. These lesions were grossly and microscopically identical with the spontaneous reticuloendothelioma. The tumorous livers proved to be a dependable and logical source for the tumor tissue needed for additional inoculations.

The carcinoma of liver parenchyma cells (C954) was originally injected subcutaneously into 12 young leaden strain mice. Six months later all the mice but 1 were discarded as negative. This mouse responded a month later by developing a nodule at the implantation site, which resembled the primary tumor and was used as the source of tumor

cutaneous tumors at the implantation site have been compared to determine the organophilic tendencies of the same tumor in different host backgrounds. Detailed observations were kept on the thoracic and abdominal structures that gave any evidence of containing foci of tumor metastases. These records always included data on the involvement of lungs, liver, kidneys, gonads, spleen and lymph nodes.

When question arose as to whether tumor C198 was present, the tissue was either sectioned and stained or tested by inoculating it into a group of susceptible leaden hosts. The former method was useful in cases of early tumor involvement in young adult mice, and both methods were used when the hosts lived long enough for new tumors to arise spontaneously. If the prepared sections were histologically identical with C198 and/or the transplanted fresh tumor tissue developed masses indistinguishable from this tumor in leaden strain hosts, the case in question was called a positive growth of C198. In this way both morphological and physiological tests were carried out.

RESULTS

A. LOCAL RESPONSE TO INJECTIONS OF TUMOR TISSUE

Transplantation of C954 and C198 into (L) strain hosts.—The general reactions of the susceptible leaden host mice to subcutaneous implants of these 2 tumors are shown in Table I. A sample of 450 mice showed that liver cell carcinoma C954 always grew progressively but remained localized at the subcutaneous site where the tumor was in-

jected. No cases of extension of this tumor to another part of the body occurred. On the other hand, the reticuloendothelioma C198 showed both internal and external manifestations of tumor growth in 100 per cent of the leaden strain mice taken as a representative sample, following subcutaneous implantations of C198, of the host-tumor relationship. The particular organs that responded to subcutaneous implants of tumor C198 are taken up later in this paper. Suffice it to note here, that only the reticuloendothelioma C198 showed a characteristic tendency to extension beyond the site of inoculation. For this reason carcinoma C954 was not analyzed further. However, tumor C198 was studied more intensively for the presence of organophilic tendencies.

TABLE 1: GENERAL RESPONSE OF LEADEN (L) STRAIN MICE TO SUBCUTANEOUS IMPLANTS OF TWO SPONTANEOUS TUMORS THAT AROSE IN ANIMALS OF THE (L) STRAIN

Tumor inoc.	Type of tumor inoculated	No. (L) mice inoc.	Subcut. response, %+	Internal response, %+
C198	Reticulo-endothelioma	1,179	100	100
C954	Carcinoma of liver	450	100	0

Inoculation of C198 into different pure strains of mice.—Animals from several inbred strains of mice were inoculated with this tumor (Table II). No mice from pure stocks, other than leaden, developed C198 following inoculation under the usual transplant conditions. These data were spread over a considerable time interval and included different tumor transplant generations.

TABLE II: LOCAL RESPONSE OF DIFFERENT TYPES OF MICE TO SUBCUTANEOUS INJECTIONS OF TUMOR C198

Strain	No. of mice	% + Subcutaneously Observed	% + Subcutaneously Expected
L	1,179	100	100
B	130	0	0
B of (L) − (B)	34	91	0
B with C198a	98	84	0
Br^a and Br^{cd}	68	0	0
dba	16	0	0
A	56	0	0
F₁ (L × B)	85	100	100
F₁ (L × Br^{cd})	42	100	100
F₁ (B × Br^{cd})	122	100	0
F₁ (B × Br^a)	60	100	0
F₁ (B × dba)	23	91	0
F₁ (B × A)	36	81	0

(L) − (B) = Mice in parabiotic union.
B with C198a = B mice inoculated with C198 that had grown successfully in B host while in parabiotic union.

Implantation of C198 into parabiotic B hosts.— This tumor has been grown in some members of the C57 black stock under exceptional conditions. Black strain mice surgically joined in parabiotic union with the susceptible leaden mice became susceptive to C198 implants. About 90 per cent

of B strain animals tested by this method grew this leaden strain tumor. An earlier publication presented this work in more detail (2).

Effect of parabiotically altered host upon C198 implant.—Once a C57 black mouse had progressively grown C198, while the mouse was under some influence activated by parabiosis, the tumor itself often showed physiological evidence of a change. This became evident when fragments of subcutaneous masses of C198 tumor were taken from positive parabiotic C57 black mice and injected into the normal untreated C57 black strain animals. Many black strain mice responded to this altered tumor, which will be called C198a. Table II shows that 84 per cent of such mice were susceptive to inoculations of C198a. No histological difference has been observed between C198 and C198a.

Inoculation of C198 into hybrid hosts.—As was expected, all first generation hybrid mice between the susceptible leaden strain and other pure strains of mice grew implants of tumor C198 from leaden donors. The data on two such crosses can be seen in Table II. All of these hosts had subcutaneous masses.

Both parents of hybrids from B mice and strains other than L were resistant to C198. Based on the genetic theory of transplantation, therefore, the expected response to injections of this tumor was very different from the data (5) observed. Nearly all these hybrids were susceptible to the tumor when it was injected subcutaneously.

In 4 crosses between C57 black mice and other resistant strains, 80 to 100 per cent of the first generation hybrids were positive to inoculations of the original C198 tumor (Table II). Leaden hosts were used as donors for all the C198 tumor material inoculated into these hybrids.

TABLE III: METASTASES OF SUBCUTANEOUS IMPLANTS OF C198 TO INTERNAL ORGANS OF DIFFERENT HOST-TYPES

Strain	Autopsied with + subcut.	Percentage with internal metastases
L	71	100
B of (L) − (B)	26	62
B with C198a	55	2
F₁ (L × B)	45	100
F₁ (L × Br^{cd})	42	100
F₁ (B × Br^{cd})	114	100
F₁ (B × Br^a)	46	100
F₁ (B × dba)	17	65
F₁ (B × A)	21	59

B. SYSTEMIC RESPONSE TO SUBCUTANEOUS TRANSPLANTS OF C198

1. *General systemic response.*—Data from the pure and hybrid mice autopsied after growing C198 subcutaneous implants are shown in Table III. The percentages of host mice that showed

metastases of this tumor to some internal organs were tabulated. The findings for specific organs in different host types are given in detail in Tables IV and V.

2. Specific systemic responses. Metastases of C198 in the L and B strains.—Table IV shows the internal sites that were grossly involved by tumor C198 after this neoplasm had been successfully implanted into the right axillary subcutaneous tissues. In the leaden hosts the liver always de-

about half the mice exhibited extensive growths of C198 in their spleens. Only a few of these hybrids had ovarian and nephric metastatic nodules of the tumor C198.

The C57 black strain mice were crossed with several pure stocks besides the leaden mice and most of these individuals developed masses at the site of tumor implantation (Table II). The organophilic tendencies observed in mice with these implants are shown in Table IV. When B mice

TABLE IV: ORGANOPHILIC TENDENCIES OF METASTASES OF C198 IN VARIOUS HOST TYPES BEARING SUBCUTANEOUS IMPLANTS OF TUMOR

Strain	Autopsied with subcut.	Percentage with internal metastases	Liver % +	Lungs % +	Kidneys % +	Spleen % +	Ovaries % +	Lymph nodes % +
L	71	100	100	76	0	65	0	6
B of (L) − (B)	26	62	58	23	0	15	0	12
B with C198a	55	2	2	0	0	0	0	0
F₁ (L × B)	45	100	100	91	7	51	17	20
F₁ (L × Br^cd)	42	100	100	48	98	0	100	24
F₁ (B × Br^cd)	114	100	77	49	91	11	89	43
F₁ (B × Br^a)	46	100	50	76	98	15	96	63
F₁ (B × dba)	17	65	18	12	18	29	0	41
F₁ (B × A)	21	59	30	11	8	15	0	19

veloped tumorous foci. Also 76 per cent of the lungs showed typical, hemorrhagic areas containing C198 tumor cells and 65 per cent of the spleens had one or more white tumor nodules.

When the C57 black strain hosts were in parabiotic combination with leaden strain mice autopsies of the C57 black strain mice with subcutaneous masses showed that 58 per cent had liver nodules; 23 per cent, lung involvement; and 15 per cent, tumors in the spleen. However, mice of this same black strain showed but very slight evidence of organophilic tendencies when they were not in parabiotic union but received and grew implants of the altered tumor, C198a. As stated previously, this tumor became changed during growth in a C57 black parabiotic host.

Metastases of C198 in F₁ hybrids.—First generation hybrid mice, where one parent was from the leaden stock, developed 100 per cent tumor metastases in their livers following subcutaneous inoculations with C198. These findings on metastases were identical with those for the leaden stock. Many of these hybrids showed pulmonary foci of this tumor. In addition to this nearly 100 per cent of the F₁ (L × Br^cd) of both sexes had multiple metastatic tumor nodules in their kidneys and all the females had ovarian metastases of C198. The ovaries were not multinodular but consisted of one rounded mass frequently more than 1 cm. in diameter. The male gonads were not grossly involved by C198. Here the spleen did not show a single metastatic tumor nodule. However, in the F₁ (L × B) hybrids with the same treatment

were crossed to Br^cd mice the findings in the internal organs of the F₁ hybrids, following the subcutaneous implants of C198, were very much like those observed in the F₁ (L × Br^cd) mice except that fewer animals showed liver metastases. Nearly all these and the F₁ (B × Br^a) hybrids developed metastases in the form of large tumorous ovaries and multiple white tumor nodules in their kidneys. On the other hand, F₁ (B × dba) and F₁ (B × A) hybrids had no cases of tumor invasion of the ovaries and a relatively small percentage of these hybrids has C198 nodules in their kidneys. (Table IV).

Hepatic response to C198 metastases.—The livers of mice with foci of C198 did not present the same gross characteristics in all host animals. The response of this organ is further analyzed in Table V. Metastatic tumors of C198 sometimes formed discrete white nodules (Nod.) when present in the liver, or the organ might be swollen and hemorrhagic (S. H.) with a diffuse growth of tumor cells but no distinct nodules. Sometimes a liver showed a swollen, hemorrhagic condition with tumor nodules. However, each type of host usually had liver metastases that were fairly characteristic for that group of animals. Table V shows that all the leaden mice had liver metastases. Nearly all these livers were swollen, hemorrhagic and had diffuse growths of metastatic tumor cells. Approximately one-third had distinct, multiple, small tumor nodules. About half the C57 black mice that were in parabiotic union with leadens, or B of (L) − (B), and that grew subcutaneous

masses produced C198 nodules in the liver. These nodules were few in number and the swollen, hemorrhagic appearance was rare. The B mice growing C198a showed almost no tendency for C198a metastasis to the liver or elsewhere.

All the F_1 hybrids of leaden mice developed C198 liver metastases and there was considerable increase in the frequency and size of the nodules over that seen in the pure leaden hosts. The F_1 hybrids of the C57 black strain crossed with stocks other than leaden showed that when C198 was present in the liver it was most frequently expressed as distinct tumor nodules.

TABLE V: RESPONSE OF PULMONARY AND HEPATIC ORGANS TO METASTASES OF C198 IN MICE GROWING SUBCUTANEOUS IMPLANTS OF TUMOR

Strain	No.	Liver Total, % +	S.H., %	Nod., %	Lungs Total, % +	H %	Nod., %
L	71	100	96	34	76	76	0
B of (L) — (B)	26	58	4	54	23	23	0
B with C198a	55	2	0	2	0	0	0
F_1 (L × B)	45	100	82	78	91	91	0
F_1 (L × Br^{cd})	42	100	55	57	48	48	0
F_1 (B × Br^{cd})	114	77	51	51	49	47	2
F_1 (B × Br^a)	46	50	24	46	76	22	65
F_1 (B × dba)	17	18	0	18	12	6	6
F_1 (B × A)	21	30	7	30	11	11	0

S.H = Swollen and hemorrhagic.
H. = Hemorrhagic.
Nod = White nodules of C198.

Pulmonary response to C198 metastases.—In the lungs the usual picture of tumor involvement was extensive, hemorrhagic foci without definite tumor nodules. F_1 (B × Br^a) hybrids were significantly different from all other types in that C198 metastases formed distinct tumor nodules in the lungs of 65 per cent of the mice autopsied, and most of these were without hemorrhagic foci (Table V). In the case of the F_1 (B × Br^{cd}) mice, 2 had lung metastases and in the F_1 (B × dba) group only 1 mouse had a C198 nodule in its lung.

Organophilic tendencies of C198.—Host mice, one or both of whose parents belonged to the leaden strain, when injected subcutaneously with tumor C198 developed liver metastases in 100 per cent of the cases. When F_1 hybrids of (B × Br^a), (B × Br^{cd}) and (L × Br^{cd}) were inoculated with C198 it was found that when one parent was descended from the Br strain the ovaries and kidneys were nearly always strikingly involved by metastases of the tumor.

The F_1 (L × Br^{cd}) and F_1 (B × Br^{cd}) hybrids with implants of C198 were very similar in the gross responses of their livers and lungs to tumor cell emboli. This is compatible with the response of the kidneys and ovaries for the same types of mice, as shown in Table IV.

DISCUSSION

The liver carcinoma, C954, remained localized at the subcutaneous implantation site until the L strain host mouse died from complications brought about by the advanced neoplastic growth. Reticuloendothelioma C198 had a marked tendency to appear in the liver and certain other internal organs of the L strain hosts after their subcutaneous implants of this tumor developed. This was referred to in an earlier paper (1). A similar condition was found in a transplantable reticuloendothelioma, called C800, that arose in a C57 black strain mouse (unpublished data). This tumor also formed metastases to the lungs, spleen and liver. Gorer (4) reported two other transplantable reticuloendotheliomas in C57 black mice. These had like preference for extension, into the liver. When tumor C198 was injected subcutaneously the mass at the implantation site in a leaden host usually remained small, and was soft and doughy. This condition was found also in the case of the three B tumors just referred to.

No published data have been found that describe tumors with as definite organophilic tendencies as have been shown here. These responses vary depending upon the type of host bearing the implants of tumor C198. The mechanical filtering-out of tumor cells by the architectural arrangement of the blood vessels, inherent in certain organs, cannot alone explain this organ-tumor relationship.

After receiving subcutaneous implants of tumor C198 all the leaden strain mice and all their F_1 hybrids showed metastases of this tumor in their livers. In all the inoculated F_1 (L × Br^{cd}) females tumor C198 metastasized to their ovaries, and in both sexes there was kidney involvement in nearly 100 per cent of the animals. On the other hand, only a small percentage of the inoculated F_1 (L × B) hybrids showed C198 tumor nodules in ovaries and kidneys. Metastatic nodules of this tumor have been found in the ovaries and kidneys of leadens but only on rare occasions. These nodules were solitary and scarcely visible. The F_1 (B × Br^{cd}) hybrids reacted to injections of C198 very much the same way as did the F_1 (L × Br^{cd}) mice except that the livers of the former did not always develop metastatic tumor lesions.

The sewing together of a resistant B to a susceptible L mouse in parabiotic union upsets the inherent refractory response of the B host mouse to implants of the L strain tumor C198. Not only did such B mice grow the tumor implants in 91 per cent of the recipients, but 62 per cent of these mice with tumors also developed internal metastases. The internal organs invaded by C198

happened to be the same as those usually involved in the leaden mice that were hosts to the tumor.

The tumor C198 was altered by its growth in the B mice that were living in parabiotic combination at the time of inoculation with this tumor. This altered line of tumor C198 was given the name C198a. B mice inoculated with C198a grew large subcutaneous masses and often lived for 4 to 8 months. However, in the 55 B mice that were hosts to C198a there was but one internal nodule found at autopsy, a tiny liver nodule. Its diagnosis as C198a was confirmed by microscopic examination. The work by Gorer (4) and our unpublished data on C57 black reticuloendothelioma C800 show that the B strain responded to transplants of its own tumors of this type by developing hepatic, pulmonary and splenic metastases. It is possible that the B mice in parabiotic union were more likely to show their inherent response to reticuloendothelioma as a tumor type, but could not as readily show this reaction to a leaden strain tumor placed subcutaneously into B mice that were not in parabiosis.

Two interesting findings are demonstrated hereby. First, the B strain mouse can be altered from its normal refractory response to the leaden neoplasm C198 if the tumor is implanted while the refractory animal is in parabiotic combination with a susceptive mouse. Second, that the growth of this tumor in such a B host, whose physiology has shifted from refractivity to susceptivity to the growth of C198, can alter the physiology of the tumor itself. Thus we can change the host, and the host in turn can change the implanted tumor that it nurtures.

SUMMARY

A carcinoma of liver parenchyma cells and a reticuloendothelioma from the liver were compared as to their organophilic tendencies. This was shown by both the local and the systemic response of the host mice that received subcutaneous injections of fragments of these two tumors. Both neoplasms originated spontaneously in leaden strain mice. Both tumors were implanted and grew successfully at the site of inoculation in all the leaden strain mice tested. Carcinoma C954 never extended beyond the right axillary subcutaneous region where the tumor tissue fragment was deposited. However, the reticuloendothelioma C198 not only appeared at the site of implantation but also metastasized to certain thoracic and abdominal organs. On the basis of this last observation a more detailed study was made of the organs involved by these metastases.

All pure strains of mice, except the leaden strain in which the tumor originated, failed to grow tumor C198 following subcutaneous injections of this tumor. The C57 black strain mice in parabiotic combination with leaden mice underwent a change so that most of these black mice were no longer refractory but became susceptible to implants of this neoplasm. Furthermore, the black strain mice physiologically changed by parabiosis were able to alter tumor C198 while it was growing in them. This physiologically altered tumor could grow in both the black and leaden mouse strain hosts. When the parabiotic black strain mice with subcutaneous growths of C198 were autopsied, 62 per cent of them had metastases to their internal organs. However, metastases to internal organs were found in only 2 per cent of the black strain mice not in parabiosis but growing subcutaneous C198a nodules implanted from the successful growths of C198 in black strain parabiotic hosts.

Several F_1 hybrid classes were made by crossing the leaden and black strains with each other and with several other mouse strains the members of which were refractory to implants of C198. The mice usually grow local masses and those with subcutaneous growths of C198 were autopsied and data were kept upon the appearance of metastatic tumor nodules in their internal organs. The most outstanding finding was that when C198 was transplanted into certain types of mice strong organophilic tendencies were shown by this tumor. Very strong hepatophilic, nephrophilic and ovariophilic tendencies for metastases of C198 were demonstrated in some host types. C198 in the F_1 hybrids from leaden mice crossed with other strains showed an hepatophilic tendency with metastases in 100 per cent of the host hybrids. This was also a characteristic finding in the pure leaden strain mice with C198 implants. No other types showed this involvement of the liver to such a marked degree.

In hybrids with one parent from the brown strain metastases from implants of C198 showed a nephrophilic tendency in 91 to 98 per cent of all mice inoculated with this tumor. Furthermore, 89 to 100 per cent of the females from these same brown strain hybrids developed massive tumor metastases in their ovaries. The other types of mice used showed either only slight or no tendency to develop tumor metastases in their kidneys and ovaries. The F_1 hybrids of Br^a crossed with B, or (B × Br^a), had white tumor nodules in their lungs usually without any accompanying hemorrhage. These were nodules of C198. The characteristic picture in all other types of mice was hemorrhagic

foci in the lungs without grossly visible nodules of tumor when pulmonary metastases of C198 occurs.

CONCLUSIONS

1. The liver carcinoma C954 always remained localized at the subcutaneous site of implantation. The reticuloendothelioma C198 usually appeared concurrently at the implantation site and in one or more of the internal organs of the host mouse.

2. Different types of mice bearing subcutaneous implants of C198 demonstrated diverse organophilic tendencies. A very strong hepatophilic, nephrophilic and ovariophilic affinity was demonstrated by the metastases of tumor C198 in certain types of host mice while other host types showed either irregular or no metastases to their livers, kidneys and ovaries. In many host animals this tumor also showed a tendency to metastasize to the lungs, spleen and lymph nodes.

3. The normal refractory response of B strain mice to C198 implants was changed by parabiotic union of B and L strain animals. In this way it was possible to change the nonsusceptible B host and to make it susceptive to inoculations of tumor C198. Such a B host in turn brought about a physiological change in the implanted tumor and thereafter the B strain mice showed a high degree of susceptibility to this "altered tumor" called C198a.

4. The appearance of tumor metastases within a specific internal organ is probably dependent upon a host-tumor interrelationship rather than upon either the tumor type or the host type alone.

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Sulfhydryl Reduction of Methylene Blue

With Reference to Alterations in Malignant Neoplastic Disease

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A significant decrease in methylene blue reducing power of plasma from patients with malignant neoplastic disease was previously reported (1). At that time it was suggested that change in a reducing group of the albumin molecule was a likely source of this alteration. Similar conclusions were reported also by Savignac and associates (7) as the result of analogous studies.

In an attempt to evaluate the effect of the sulfhydryl group on the reduction of methylene blue, a study was undertaken with various compounds of known -SH and S-S structures. In addition, an attempt was made to establish a standard method of calibration of various lots of methylene blue, so that more uniform results would be possible in the plasma reducing test.

Glutathione, cysteine hydrochloride and methionine were made up in equimolar solutions (0.0325 M) in distilled water. One cc. of glutathione was added to 0.2 cc. of 0.13 per cent methylene blue in a Wasserman tube. Similarly, 0.2 cc. of methylene blue (0.13 per cent) was added to cysteine and to methionine. The tubes were immersed in a boiling water bath and observed for time of complete decolorization.

The tube containing methionine and methylene blue failed to show any change in color in spite of continued boiling for an hour and a half. On the other hand, complete decolorization was noted in the tubes containing cysteine HCl and glutathione in 6.0 and 15 minutes, respectively.

An attempt was then made to evaluate changes in the reducing time with varying concentrations of cysteine and glutathione. Thus 1 cc. of varying concentrations of cysteine HCl was mixed with 0.2 cc. of methylene blue (0.13 per cent) and the time noted for complete decolorization. Equimolar solutions of glutathione were treated in a similar fashion. The values obtained are indicated in Fig. 1. The results indicate a linear relationship between the cysteine concentration and the reducing power, and a definite limiting value of cysteine concentration for reduction of the methylene blue. The reactions with glutathione are similar, but the

reactivity is less than half that of the cysteine. It is noteworthy also that the resultant leuco mixture did not revert back to colored methylene blue on cooling, as was the case with methylene blue reduction by plasma.

Similar relationships were investigated between cysteine and different concentrations of methylene blue. As seen in Fig. 2, similar curves are obtained, but the position of the curve on the graph varies with the concentration of the methylene blue used. It should be noted that there is no appreciable difference in the reducing time of methylene blue on varying the concentrations between 0.10 per cent and 0.2 per cent, although 0.08 per cent shows a decided difference.

Analogous findings were obtained on mixing similar concentrations of methylene blue with a plasma sample. The following reducing times were obtained when a plasma sample was used to reduce the methylene blue solutions:

Methylene blue, per cent	Reducing time, minutes
0.08	5.5
0.13	8
0.20	8

The curves depicted in Fig. 2 possess an additional feature which merits attention in relation to the results obtained with plasma, namely, the sharp break in the curves occurring after the methylene blue reducing time of 15 minutes. A similar break is found at 15 minutes when the occurrence of various reducing times of numerous plasma samples from cancer patients is plotted. These figures are based on 109 plasma samples whose reducing times fell between 13 and 25 minutes. As with the cysteine, apparently, a point is reached beyond which decrease in reducing activity is attended by marked change in the reducing time; in short, a steeper rate of change.

In view of the parallel results obtained with cysteine HCl and plasma, the cysteine solutions may be used to test the reactivity of different lots of methylene blue. Concentrations between 0.1
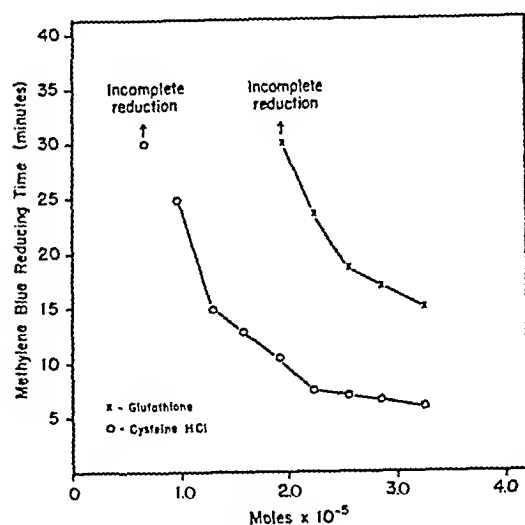
Fig. 1- Reduction of methylene blue (1 cc. of 0.13%) by cysteine HCl and glutathione
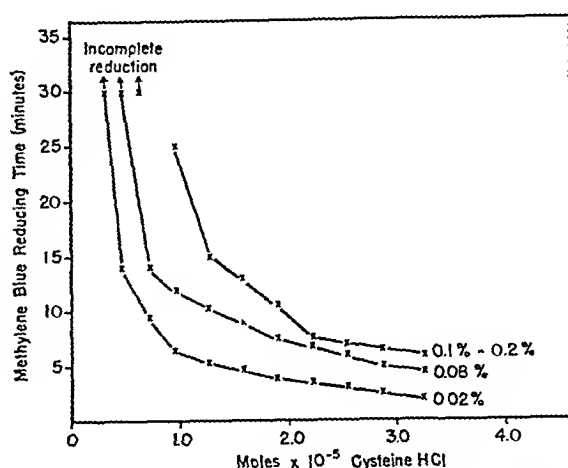
Fig. 2.- Reduction of methylene blue (0.2 cc.) of varied concentration
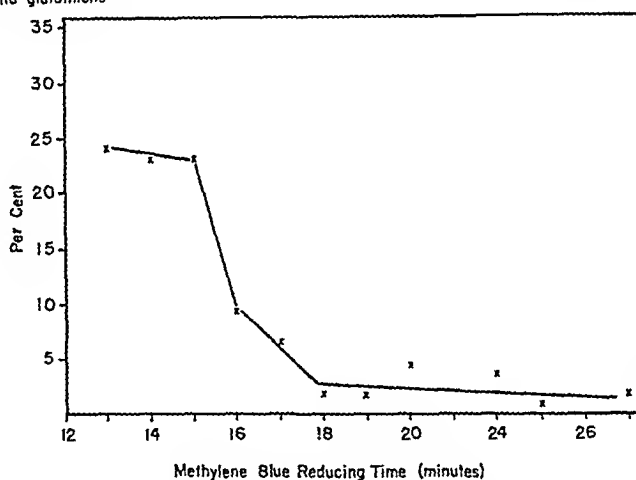
Fig. 3.- Distribution of reducing time values in 109 cancer cases

per cent and 0.2 per cent would seem to be most efficacious, since variations in this zone produce minimal changes in reducing time. Thus, a concentration of 0.13 per cent methylene blue has been adopted in evaluating the reducing power of plasma.

The apparent decrease in sulfhydryl activity in plasma of patients with malignant neoplastic disease suggested therapeutic trial of sulfhydryl compounds on such patients. Glutathione in doses of 50 to 100 mgm. was injected intravenously in several patients with varying types of malignant neoplastic diseases. A reaction of chills and fever occurred in most cases in about 30 minutes and lasted from 20 minutes to an hour. This was usually replaced by a sense of well being and relief from previous symptomatic complaints, *viz.* pain, asthenia etc. Beneficial effects usually lasted several days and could be regained by repeated injections. Similar results were obtained by injection of 25 to 50 mgm. of cysteine HCl although the initial reaction did not occur. It should be mentioned that although the symptomatic improvement might be great, there was no apparent effect on the growth of the neoplasm.

DISCUSSION

The reduction of methylene blue by cysteine and glutathione and not by methionine is an indication that, in these compounds at least, a free -SH bond is required. The more prolonged time found for glutathione (15 minutes) as compared with cysteine HCl (6 minutes) would seem to indicate that the availability or reactivity of the -SH bond may be altered by its location in the molecule. Thus in cysteine the -SH is terminal and presumably unhindered in its reactivity. In

the case of the glutathione, the internal location of the -SH bond seems to decrease its reactivity.

The reversible decrease in reducing power of plasma associated with malignant neoplastic disease might be explained on the basis of changes in the spatial configuration of the albumin molecule. Such changes would be readily reversible and would not necessitate changes in amount of total protein of -SH bonds. This is of importance since the observed decrease in reducing power is not correlated with changes in concentration of plasma proteins.

The observation that glutathione and cysteine were often efficacious in relieving the symptomatic complaints of patients with malignant neoplastic disease finds some parallel in the literature. Thus, Stern and Wilheim (8), in reviewing the relation of sulfhydryl compounds to life processes, point out that the growth inhibition of normal animals treated with various organic carcinogens could be cancelled by increased quantities of sulfhydryl compounds in the diet. This is interpreted as being due to utilization of sulfhydryl compounds in detoxification of the carcinogens and also the competitive utilization of -SH groups by neoplastic tissue itself, leaving relative deficiencies of these indispensible requisites for growth, enzyme activity and metabolism. Therapeutic use of cysteine and glutathione also has been suggested by the observation of tumor growth inhibition in some neoplasms (2, 3, 5), although no such effect was obtained in others (4).

The data presented in this and our previous study would seem to point to the importance of the sulfhydryl group as a factor in the over-all redox substrate of the body rather than as a specific tumor-inhibiting agent. One might consider this group analagous to a buffer in acid-base systems. Loss of such control would lead to suboptimal sulfhydryl potentials with attendant alterations in function of various enzyme systems. The report by Hirshfeld, Duboff, and West (6) of an inhibitory action of serum of cancer patients on the tyrosine-tyrosinase system might be explained on the basis of such alteration in optimal -SH potential.

SUMMARY

1. Methylene blue is reduced by boiling with cysteine HCl and with glutathione but not with methionine.

2. The reaction with cysteine HCl is more rapid (6.0 minutes) than the reaction with equimolar glutathione (15 minutes).

3. The reaction between cysteine and methylene blue shows a linear relationship, which may be used for calibration of different lots of methylene blue.

4. A possible relationship between the spatial configuration of the albumin molecule and the decrease in reducing power of plasma in the presence of malignant neoplastic disease is discussed.

5. Effects of the administration of cysteine and glutathione on patients with malignant neoplastic diseases are reported.

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Abstracts

Reports of Research

Azotoluene Bladder Tumours in Rats. Strombeck, J. P. [Surg. Clin., Lund, Sweden] *J. Path. & Bact.*, 58:275-278. 1946.

Azotoluene given by the mouth to rats does not produce neoplastic changes in portions of the bladder transplanted to the liver.—E. L. K.

The Induction of Mammary Carcinoma in "IF" Mice by Cutaneous and Intraperitoneal Administration of Methylcholanthrene. Orr, J. W. [Dept. Exper. Path. and Cancer Research, Univ. of Leeds, Leeds, England] *J. Path. & Bact.*, 58:589-592. 1946.

The IF strain of mice appears to be resistant to the development of mammary cancer, only one possible mammary carcinoma having been found in an untreated female. On the other hand, fortnightly cutaneous applications of methylcholanthrene dissolved in almond oil induced mammary carcinoma, frequently multiple, in 30 of 37 females of the strain. Tumors developed in all female mice surviving this treatment for 126 days or more, the mean induction time being approximately 140 days. Intraperitoneal injections of methylcholanthrene in sesame oil induced mammary carcinoma in 1 of 12 IF mice after 105 days, the maximum survival period of the group being 113 days.—A. II.

Measurement of the Photodynamic Effect of Cancerogenic Substances with Biological Indicators. Matoltsy, G., and Fábián, Gy. [Biol. Research Inst., Tihany, Hungary] *Nature*, 158:877. 1946.

When *Drosophila* flies were fed in the dark on a standard diet containing carcinogens (1 mgm. per 13 gm.), the hydrocarbon could be detected in the organs and cells of of the larvae by fluorescence microscopy. The survival time after raying with ultraviolet light was found to be as follows: benzpyrene treated for 6 minutes 30 seconds; methylcholanthrene 13 minutes 35 seconds; dibenzanthracene 19 minutes 34 seconds; controls *i.e.* larvae bred on standard diet, survived 39 minutes 51 seconds. (No mention is made of controls which should have been made where fluorescent noncarcinogenic hydrocarbons were added to the diet.)—I. H.

Test of a Cancerogenic Substance in Respect to the "Non-disjunction" Frequency of the X-Chromosomes in Drosophila. Fábián, Gy., and Matoltsy, G. [Biol. Research Inst., Tihany, Hungary] *Nature*, 158:911-912. 1946.

Drosophila larvae and adult flies were fed on a diet containing benzpyrene, and the hydrocarbon could be detected by fluorescence microscopy in the larvae, eggs and ovaries. Some tests have been made on the effect of carcinogenic substances on *Drosophila* in respect to

mutation frequency (Auerbach, C., *Proc. Roy. Soc. Edinburgh*, 60:164; Friedrich-Freksa, H., *Biol. Zentralbl.*, 60:498). The results showed that the mutation rate does not increase following applications of carcinogenic chemicals. The cultures were kept in darkness, while the primary nondisjunction was investigated. Bar males and white females were used from 2 inbred stocks which had been kept pure for 5 years, and so may be considered well-balanced stocks from the point of view of modifying factors. In this standard arrangement the frequency of exceptions was 1:500, or 0.2 $\pm$ 0.05% without benzpyrene. There was not a significant difference between the control and the treated cultures in respect to the number of exceptional offspring. In a succeeding test, the adult females were fed entirely on food containing benzpyrene, and in this case, using the fluorescence microscope, it was evident that the benzpyrene was also present in the eggs when they were laid. In this test the ratio of exceptions was 1:2,300, or 0.04 $\pm$ 0.03%. The standard errors showed a statistically significant difference (0.16 $\pm$ 0.059) in the negative direction between controls and the second test. None of the treatments used gave any detectable increase in the nondisjunction frequency; but treatment with benzpyrene decreased the number of exceptional flies, that is, the mutation rate. The reason for this is not known.—I.H.

Uncoordinated Growth in Paramecium Induced by "Gammexane." Lloyd, L. [Univ. of Leeds, Leeds, England] *Nature*, 159:135. 1947.

The γ-isomer of hexachlorocyclohexane produces in *Paramecium* large multinucleated forms, as the late J. C. Mottram observed, after treating these organisms with carcinogenic hydrocarbons.—E. L. K.

Induction of Glandular Carcinomas of the Prostate in the Mouse. Horning, E. S. [Imperial Cancer Research Fund, London, England] *Lancet*, 2:829-830. 1946.

The epithelium from either the anterior or dorsal lobes of the prostate of 6 months old Strong A mice was cut into strips, wrapped around some crystals of 20-methylcholanthrene, and implanted subcutaneously in male mice of the same strain. Three such grafts can be made in one host. In 10½ weeks small tumors appear; of 11 tumors 10 were glandular and 1 squamous cell in nature. Some are growing in the eighth grafted generation, and show considerable secretory activity.—E. L. K.

Significance of Carcinogenic Agents. Šula, J. *Časop. lék. česk.*, 80:698-700. 1941.

The author discussed the similarity of the chemical structure of methylcholanthrene and bile acids and the

possibility of the production of methylcholanthrene from some products of the sterol metabolism in the organism. The relationship to the sex hormones is also considered. —B. S.

Relations of Steroid Hormones and Anhydro-Hydroxy-Progesterone to Fibromatosis. IGLESIAS, R., and LIPSCHÜTZ, A. [Nat. Health Service and Univ. of Chile, Santiago, Chile] *Lancet*, 2:488-490. 1946.

Anhydro-hydroxy-progesterone has an inhibitory action upon uterine fibroids, and to a lesser extent upon other abdominal fibroids, induced in castrated female guinea-pigs by 2-estradiol. No masculinizing action was observed. The authors suggest clinical trials of this compound, together with small quantities of testosterone propionate, in cases of uterine fibroids.—E. L. K.

Genes and Nucleoproteins in the Synthesis of Enzymes. SPIEGELMAN, S., and KAMEN, M. D. [Mallinckrodt Inst. of Radiol., and Washington Univ. Sch. of Med., St. Louis, Mo.] *Science*, 104:581-584. 1946.

Yeast cells were grown in a medium containing radioactive phosphorus (P^{32}) for 48 hours, at which time all fractions contained P^{32} in equilibrium with the P^{32} in the medium. The cells were then washed, and were allowed to ferment glucose anaerobically in absence of nitrogen. Under these conditions no protein synthesis or cell division occurred, and there was no loss of P^{32} from the nucleoprotein fraction of the cells even though the non-nucleoprotein, acid soluble phosphorus was rapidly equilibrated with that of the medium. The same result was obtained in the presence of nitrogen when cell division was inhibited by sodium azide or dinitrophenol. When, however, the cells were allowed to synthezise protein and to divide, it was found that large amounts of P^{32} were lost from the nucleoprotein, indicating a flow of phosphate from this fraction in the course of synthesis. The same flow of P^{32} from nucleoprotein was observed when the cells were induced to form a new enzyme in the course of adaptation to a new substrate.

These results plus other evidence reviewed in this paper have led the authors to suggest that nucleoproteins are the controlling elements in protein formation. They hypothesize that genes continually give off at different rates partial replicas of themselves to the cytoplasm. These replicas are nucleoprotein in nature, possess in varying degrees the capacity for self reproduction, and together determine the types and amounts of proteins and enzymes synthesized. Furthermore, it is supposed that a competitive balance exists among the various cytoplasmic nucleoproteins, that this balance may be changed by changing certain conditions under which the cells live, and that such altered relationships among the cytoplasmic nucleoproteins may be passed on to subsequent cell generations. This theory thus attempts to bring together under a unified interpretation (1) Mendelian, or "gene" controlled inheritance, (2) cytoplasmic inheritance, (3) cellular differentiation, and (4) enzymatic adaptation. The origin of cancer as a sudden heritable change in somatic cells, analogous in many ways to enzyme adaptation or cellular differentiation, also comes within the scope of this theory.—R. B.

Ultra-Violet Absorption in Living and Dead Cells. BRUMBERG, E. M., and LARIONOW, L. TH. [Optical Inst., and Central Roentgenological, Radiological and Cancer Inst., Leningrad, U. S. S. R.] *Nature*, 158:633-664. 1946.

An ultraviolet microscope equipped with a special achromatic objective (aperture 0.5) was employed for photographing living tissue cultures. The source of light was a high-pressure quartz mercury lamp. All radiations except of wave length 254-275 $\mu\mu$ were eliminated by filters. Focussing was performed by ordinary lighting, so that no ultraviolet rays reached the cells prior to their being photographed. When living mouse mammary carcinoma cells and mouse and chick fibroblasts were examined, only nucleoli and the cytoplasm of cancer cells revealed moderate absorption in the region 254-275 $\mu\mu$. The cytoplasm of cancer cells vitally stained with neutral red lost its capacity for absorption. Preliminary exposure of cultures to direct ultraviolet irradiation (without filters) for 2 minutes resulted in definite cytological changes. Nuclei then acquired the property of absorption and photographs presented the same appearance as those published by Caspersson.

It is suggested that desoxyribonucleic acid is contained in the nuclei of living cells in a form which does not absorb ultraviolet rays of wave length about 260 $\mu\mu$, and that absorption only occurs in injured and dead cells.—R. J. L.

Studies in Vitro on Cellular Physiology. The Effect of X-rays on the Survival of Cells. SCHREK, R. [Veterans Administration, Hines, Ill.] *Radiology*, 46:395-410. 1946.

Cellular suspensions of thymus, spleen, bone marrow, and testes of rabbits and from leukocytes of normal and leukemic blood of men were irradiated with 20 to 5,000 r and incubated at 37° C. for 1 to 7 days. Periodic examination of cell counts and stained smears were made and graphed as to the 50 and 10 % survival time. A dose of 1,000 r to thymic and splenic suspensions produced no perceptible change in 3 hours but produced a relatively rapid decrease in the unstained cell counts after this period. Irradiated leukocytes from normal and lymphocytic blood had a shorter survival time than non-irradiated blood. Suspensions from myelogenous leukemia, bone marrow and testes showed no effect of irradiation. Tests made under anaerobic conditions showed no perceptible decrease in the number of eosin-resistant lymphocytes which had been irradiated.—R. E. S.

Mechanism of Radiation Effects Against Malignant Tumors. WARREN, S. [Boston, Mass.] *J. A. M. A.*, 133: 462-463. 1947.

It is the radiant energy absorbed by the tissue or cell that is effective, not that which is delivered to it. The first noticeable effect on cells is interference with mitosis —both diminution in mitotic activity and the appearance of chromosomal abnormalities followed by vacuolization and swelling of the cytoplasm. There are concomitant effects produced on the connective tissue stroma and blood vessels within the irradiated area. The vascular endothelium is damaged inducing thrombosis. Hence local impairment of circulation in the tumor and tumor bed results. Hyaline changes leading to the formation of dense

collagen thus act as an added barrier to the metabolic activities of tumor cells and hinders their spread to adjacent structures. The same general order of sensitivity prevails among the tissues exposed to radiation by an atomic blast as by therapeutic means.—M. E. H.

Biochemical Aspects of Over-Activity of the Adrenal Cortex. SCOWEN, E. F., and WARREN, F. L., *Proc. Roy. Soc. Med.*, 40:39-43. 1946

Adrenal cortical carcinoma in females of all ages is associated, except in rare cases, with increased excretion of 17-ketosteroids; an excessive excretion of dehydroisoandrosterone is characteristic of these tumors. Owing to the rarity of adrenal carcinoma in males it is not yet possible to say whether the sterol metabolism is the same in them as in female patients.—E. L. K.

The Disposition of C[14] in Bone. BLOOM, W., CURTIS, H. J., and McLEAN, F. C. [Univ. of Chicago, Chicago, Ill., and Monsanto Chemical Co., Dayton, Ohio] *Science*, 105:45. 1947.

C[14] injected as carbonate into rats was deposited in bone primarily in non-growing areas, and persisted there apparently undiminished for the 4 months' duration of the experiment. In contrast, the C[14] deposited in the liver and kidney remained there for only 2 weeks. The authors suggest that the health hazards involved in working with this radio-isotope of carbon should be studied with special reference to the possible development of bone tumors. —R. B.

A Plan for Analysis of the Biologic Factors Involved in Experimental Carcinogenesis of the Thyroid by Means of Radioactive Isotopes. HERTZ, S. [Boston, Mass.] *West. J. Surg.* 54:487-489. 1946.

A plan is presented for the analysis of experimental carcinogenesis in animals, drawn up from experience to date, from the study of normal and pathologic thyroid physiology, chemistry and therapeutics by means of radioactive isotopes of iodine. It is hoped that a logical theory of carcinogenesis and an understanding of important preventive and therapeutic factors may be evolved.—M. E. H.

The Effect of Certain Azo Dyes upon the Storage of Riboflavin in the Liver. GRIFFIN, A. C., and BAUMANN, C. A. [Univ. of Wisconsin Coll. of Agric., Madison, Wis.] *Arch. Biochem.*, 11:467-476. 1946.

Since riboflavin is known to counteract the carcinogenicity of several of the azo dyes, investigations were carried out to determine whether the effect of a given dye on hepatic riboflavin might parallel its potency in inducing liver tumors. Such a parallelism was observed. The addition of many carcinogenic azo dyes to the diet of rats caused some decrease in the riboflavin content of the liver. The decrease appeared to be roughly equivalent to the carcinogenicity of the dye: *m'*-methyl-*p*-dimethylaminoazobenzene was most effective, *p*-dimethylaminoazobenzene and *p*-monomethylaminoazobenzene were fairly effective, whereas *o'*-methyl-*p*-dimethylaminoazobenzene. *p'*-methyl-*p*-dimethylaminoazobenzene, amino-

azotoluene, aminoazobenzene, or azobenzene had little or no effect.

More riboflavin was stored in the liver when the basal diet contained 24% of casein than when 12% was fed. The relative effects of the carcinogens were essentially the same on both diets.

The presence of carcinogenic azo dyes in the diet resulted in a decreased food intake, but this was not responsible for the impaired vitamin storage. Rats fed restricted amounts of the control diet free from the dye showed only a slight decrease in the total amount of riboflavin per liver while the concentration of vitamin per gm. of liver tissue was usually higher than in rats fed *ad libitum*.—Authors' abstract.

The Influence of Liver L. casei Factor on Spontaneous Breast Cancer in Mice. LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., and KERESZTESY, J. C. [Mount Sinai Hosp., New York, N. Y.] *Science*, 104:436. 1946.

Among 28 Rockland mice injected intravenously daily with 5 μgm. of fermentation *L. casei* factor over a period of 4 to 6 weeks there were 11 in which the mammary cancer regressed completely; after 100 days 23 mice were still alive. Among 39 mice injected in the same way with a daily dose of 5 μgm. of liver *L. casei* factor there was only 1 complete regression, and the mean life span was 75 days. Lung metastases in this group were more numerous than among the controls. Thirty-one additional mice were treated with daily doses of 100 μgm. of liver *L. casei* factor. In this group there were no regressions; in fact the mammary tumors grew faster than did those of the controls. The mean life span was 55 days. Among the 71 control mice there were no regressions and the mean life span was 74 days.—R. B.

Les facteurs antiblastiques d'origine alimentaire. [The Anti-Blastic Factors of Dietary Origin.] MAISIN, J., and POURBAIX, Y., *Bull. Assoc. franç. p. l'étude du cancer*, 29:223-251. 1940.

A review with 31 references is presented. Protocols are presented of 13 experiments, previously reported by Maisin and his colleagues, showing the cancer-inhibiting action of various foods, including fresh beef heart, whole rye flour, and fresh baker's yeast.—G. H. H.

Tumors in Intrasplenic Ovarian Transplants in Castrated Mice. LI, M. H., and GARDNER, W. U. [Yale Univ. Sch. of Med., New Haven. Conn] *Science*, 105:13-15. 1947.

Ovaries grafted into the spleens of castrated mice (A strain, C₃H strain, C₃H × A F₁ hybrids) developed tumors 130 to 346 days after grafting. Among 21 castrated males there developed 5 (possibly 7) granulosa cell tumors plus 1 mixed tumor. Among 33 castrated females, in which the intrasplenic ovarian grafts showed no adhesions to adjacent structures, there developed 4 luteomas plus 7 mixed tumors. Among 19 additional castrated females, in all of which the intrasplenic ovarian grafts showed vascularized adhesions. there developed only 1 luteoma in a mouse that had irregular estrus cycles during the latter part of the experimental period. No tumors developed from control grafts of ovaries into

the subcutaneous tissue of castrated females, normal and castrated males, and into the testes of normal males; except for one questionable granulosa cell tumor developing from a subcutaneously transplanted ovary in a castrated female mouse.

The development of tumors from intrasplenic ovarian grafts appears to be due to an increased production of gonadotropins in the host following castration. Grafts placed in the spleen are exposed to these hormones before they reach the liver where they appear to be inactivated. —R. B.

Hypervolemia in Mice Bearing Transplantable Granulosa Cell Tumors. FURTH, J., and SOBEL, H. [Cornell Univ. Med. Coll., and New York Hosp., New York, N. Y.] *Science*, 105:41. 1947.

Mice bearing transplanted granulosa cell tumors were previously found to have livers and other abdominal organs congested with blood. In the present study these animals were shown to have striking increases in blood volume. By the exsanguination-perfusion technic, the blood volume, in per cent of body weight, was found to be 13.6 for the experimental mice compared with 5.2 for the controls. By the Evans blue (T-1824) dye technic, the values were 34.3 and 10.9 respectively. The hematocrit values of the blood of the experimental mice were normal, indicating a large increase in both plasma and red cells. Control mice bearing any of four other types of tumors showed no increase in blood volume.

It is suggested that the granulosa cell tumors may secrete a substance causing the hypervolemia, and that this may be accompanied by the appearance of excessive amounts of a vaso-depressor material which would account for the observed vasodilation associated with the blood volume increase.—R. B.

The "Cytogenetics" of Black and White Guinea Pig Skin. BOILLINGHAM, R. E., and MEDAWAR, P. B. [Univ. of Oxford, Oxford, England] *Nature*, 159:115-117. 1947.

Black guinea pig epidermis "infects" and thus blackens white epidermis upon which it is grafted. This is due, not to invasion and displacement of white cells by black, but to some agent which enters the white cells and brings about a permanent heritable change that causes them and their descendents to become and remain black.— E. L. K.

Interpretive Morphology. MOORE, R. A. [Washington Univ. Sch. of Med., St. Louis, Mo.] *Proc. Inst. Med. Chicago*, 16:306-312. 1947.

The theme of the lecture was that pathologic anatomy can take a place beside experimental pathology as a dynamic science. In the mind of the observer of morphologic changes, there may be the consideration of the contraction of muscles, the secretion by cells, the action of enzymes, and the elaboration effects of hormones, as well as parthogenetic development of the sex cell. Examples of the histogenesis and development of various tumors of the genitourinary tract served as examples.— M. E. H.

General Pathology of Tumors of Endocrine Glands. KARSNER, H. T. [Western Reserve Univ. Sch. of Med., Cleveland, Ohio] *Bull. New York Acad. Med.*, 22:503-510. 1946.

A general review of tumors of endocrine glands including brief discussions of the genesis, morphology, chemical components and physiological manifestations of these tumors.—M. T.

Protective Action of Desoxycorticosterone Acetate Against X-Ray Induced Liver Changes. ELLINGER, F. [Long Island Coll. of Med., Brooklyn, N. Y.] *Science*, 104: 502-503. 1946.

This paper reports a study of desoxycorticosterone acetate as a remedy for radiation sickness, this sterone being selected because it counteracts effects of histamine or histamine-like substances which may be the cause of radiation sickness.

One hundred and sixty-eight male white mice were given 500 and 1,000 r/air in one exposure or in fractions of 100 r daily. Seventy-nine of these animals were given in addition daily subcutaneous injections of 0.25 or 0.50 mgm. desoxycorticosterone acetate (in oil), the total dose varying between 2.5 and 8.0 mgm. The most striking effect of the sterone was a reduction in the amount of sudanophile fat appearing in the liver as a result of the irradiation. There was also a slight decrease in mortality among the injected animals, but no definite alteration in the radiation effects on spleen and bone marrow.—R. B.

A New Method of Making Radon Ointment. CARDENAS, L., and WEATHERWAX, J. L. [Philadelphia Gen. Hosp., Philadelphia, Pa.] *Radiology*, 46:381-384. 1946.

A method of making radon ointment by impregnating charcoal or other adsorbent with radon is described.— R. E. S.

Les tumeurs mammaires bénignes chez l'animal. Leur intérêt biologique. [Benign Mammary Tumors of Animals. Their Biological Aspect.] ROUSSY, G., and GUÉRIN, M. [Cancer Inst., Paris, France] *Presse méd.*, 52:313-314. 1944.

A review.—C. A.

Some Notes on the Cancer Problem. KOSOLAPOFF, G. M. [Monsanto Chemical Co., Dayton, Ohio] *Science* 104:491-492. 1946.

In a preceding letter to *Science* (104:167. 1946.) K. S. Pilcher proposed that the cancer problem should be attacked through a large-scale, well planned, completely co-ordinated program directed by a group of experts following, as examples, the successful wartime programs in atomic physics, penicillin production, and so on. In the present letter Kosolapoff points out that the successful wartime programs were based on discoveries already in existence, which indicated a clearly defined line of approach. No such clear-cut line of approach to a solution of the cancer problem can be said to exist at present. Therefore, while promising fields of investigation now known should be supported in co-ordinated research, there should be at least equal support given to independent groups of investigators not tied *to any definite* approach.—R. B.

Clinical and Pathological Reports

Clinical investigations are sometimes included under Reports of Research

DIAGNOSIS—GENERAL

The Roen'gen Diagnosis of Pancreatic Cyst. HOLT, J. F. [Univ. of Michigan, Ann Arbor, Mich.] *Radiology*, 46:329-333. 1946.

A correlation of clinical and roentgen findings will often enable the roentgenologist to diagnose pancreatic cyst, particularly cysts arising in the tail of the pancreas. In such cases anterior displacement of the stomach and a smoothly rounded indentation relatively high on the greater curvature of the stomach associated with a rounded, ballotable, freely movable mass in the left upper quadrant of the abdomen permit a reasonable assurance of a correct diagnosis.—R. E. S.

The Vaginal Smear. Practical Applications in the Diagnosis of Cancer of the Uterus. MEIGS, J. V. [Vincent Memorial Hosp., Boston, Mass.] *J. A. M. A.*, 133:75-78. 1947.

The diagnosis of cancer of the cervix as well as cancer of the endometrium can be made with a high percentage of accuracy by the vaginal smear method. It is of tremendous value in the routine screening of patients either in the clinic or in the hospital. Early cases of cancer, *i.e.* "cancer *in situ*," can be diagnosed by this method. The positive vaginal smear indicates the necessity of further examination as well as the need for biopsy specimens. In this way the pathologist may confirm the observation disclosed by the smear. Although smears obtained from the vagina may be an office procedure, the interpertation of the sample should be made by a trained cytologist, one familiar with this method of diagnosis.—M. E. H.

THERAPY—GENERAL

Treatment of Basal Cell Epithelioma by Injection of Tissue Extracts. A Preliminary Report. AMERSBACH, J. C., WALTER, E. M. and SPERTI, G. S. [New York Post-Graduate Med. Sch. and Hosp., New York, N. Y.] *Arch. Dermat. & Syph.*, 54:119-132. 1946.

A brief review is presented of literature pertinent to the treatment of cancer by non-destructive methods in which tissue extracts are used to induce tumor resistance in laboratory animals. The close relationship between normal and cancer cell metabolism and the resistant state is pointed out. In this series 21 patients with basal cell epitheliomas were treated with intradermal injections of spleen or liver extracts. Fourteen patients showed complete regression of the lesions, in 1 no improvement was seen, and the remainder, still under observation, were steadily improving.—H. H.

Radiation Therapy Conference. CANTRIL, S. T., and BUSCHKE, F., Editors. [Swedish Hosp. Seattle, Wash.] *West. J. Surg.*, 54:369-370. 1946.

The largest palliative accomplishment of roentgen therapy in breast cancer is in the retardation of bone metastases and the alleviation of pain which it gives.—M. E.H.

Modern Developments in Radiotherapy and Their Practical Applications. CHARTERIS, A., and PARK, S. D. S., *Glasgow, M. J., Trans. Roy. Med. & Chir. Soc. of Glasgow*, 21:35-45. 1944.

A review.—E. L. K.

Organisation of Cancer Treatment. *Brit. M. J.*, 1:921-922. 1946.

The 16th Annual report of the National Radium Trust and Radium Commission covers the period 1944 to 1945 and contains hitherto unpublished observations in the annual reports of the war years. By the authority of the Cancer Act (1939) the commission has been increased from 10 to 17 members and is now in charge of radioactive materials other than x-rays and radium. Moreover, the Commission has advised local authorities with regard to organisation of cancer services. It was recommended that radiotherapy should be carried out only by experienced persons; that physicists should be an integral part of the staffs; and that radiotherapy centers should be limited in number and possess suitable apparatus and personnel to serve a population of 2 million. The Commission has also drawn up a system of records which is now in use for cancer patients.—M. L.

RADIATION

The Treatment of Late Post-Irradition Ulcers with Radon Ointment. LOW-BEER, B. V. A., and STONE, R. S. [San Francisco, Calif.] *Radiology*, 46:149-158. 1946.

Twenty-eight patients with post-radiation ulcers were treated with radon ointment containing 36 μc. of radon per gm. of vaseline. Details of the technic for its use are given. Alleviation of pain and healing of the ulcers occurred when there was no extension of the ulcer to bone and no malignant change. The authors consider radon ointment the preferable nonsurgical method of treatment of postirradiation ulcer.—R. E. S.

Energy Absorption in the Trunk in the Radium Treatment of Breast Cancer by Interstitial and Surface Applicator Methods. WILSON, C. W. [Westminster Hosp., London, England] *Radiology*, 46:364-372. 1946.

The integral dose, *i.e.*, the total energy absorbed in the trunk, has been calculated for conditions corresponding to those of interstitial and surface radium therapy of cancer of the breast. Graphs were then made showing: (1) variation of integral dose per mgm. hr. with positions of radium along the trunk; (2) variation with distance of radium beyond the trunk; and (3) variation with distance from the axis of the trunk, and integral dose for surface applicators. Correlation of integral dose with reaction of lymphocytes during interstitial radium therapy of cancer

of the breast indicates a regular behavior. It is felt that site and dose rate are important factors in assessing the clinical importance of integral dose.—R. E. S.

Protection Measurements of Lead-Shielded Radium. BRAESTRUP. C. B. [Dept. of Hosps., New York, N. Y.] *Radiology*, 46:385-390. 1946.

Most protection measurements are based on studies obtained with narrow collimated beams but in most instances scattered radiation from neighboring objects is an important contributing factor to the dose received by radium workers. This is borne out by measurements made with the narrow beam and wide beam. Scattered radiation may add materially to the calculated dosage rate. Lead protection shields also depend on the arrangement of the walls and shape of the shield. Better protection may be obtained by limiting the cross-section of the beam or by locating the radium at a distance from scattering objects.—R. E. S.

SKIN AND SUBCUTANEOUS TISSUES

Keloid Formation in Both Ear Lobes. WEAVER, D. F. [Detroit, Mich.] *Arch. Otolarying.*, 44:212-213. 1946.

The recurrence in a Negro woman of large keloids, which developed after the ear lobes were pierced for the insertion of earrings, were apparently prevented by roentgen rays.—C. R. N.

Adenomyoepithelioma of Sweat Gland. Report of a Case. HARTZ, P. H. [Public Health Service, Curaçao, N. W. I.] *Am. J. Clin. Path.*, 16:385-390. 1946.

A case report.—S. H. D.

Small Tumours of the Skin. MACKECHNIE, H. A. [Vancouver, B. C.] *Canad. M. A. J.*, 56:56-58. 1947.

The author discusses tumors of the skin both in the neoplastic sense and those resulting from some inflammatory process giving rise to hyperplasia or hypertrophy of tissue. All lesions suspected of malignancy should be subject to a biopsy for confirmation of the diagnosis.—M. E. H.

Tumors of the Skin. A Review of Recent Literature. Part I and Part II. BEERMAN, H. [Philadelphia, Pa.] *Am. J. M. Sc.*, 211:480-504, and 212:479-505. 1946.

A full review of the subject with 4 pages of references. —M. T.

Cancer of the Skin. A Statistical Report. ULLMANN, H. J. [Santa Barbara, Calif.] *Radiology*, 46:279-281. 1946.

During a period of 23 years the author saw 1,347 cancers of the skin, treating 1,269 by radiation and 78 by surgery. Four hundred and forty-three of those treated by radiation were followed 2 or more years and showed 25 recurrences. No treatment factors are included in this statistical report.—R. E. S.

NERVOUS SYSTEM

A Preliminary Report of the Study of 200 Autopsy Cases at the Eastern State Hospital with Special Emphasis on Neuropathology and Brain Tumor in Old Age. ZFASS, I. S., and RIESE, W. [Eastern State Hosp., Williamsburg, and Med. Coll. of Va., Richmond, Va.] *Virginia M. Monthly*, 71:281-287. 1944.

The report concerns itself with 205 patients autopsied during the 5 year period 1937 to 1942. Among these 65% were over 60 years of age and 83% over 50. There were 10 (4.9%) neoplasms among the 205 autopsies; 7 occurred in patients past 50 and 4 of the 7 were 64, 70, 71 and 76 years of age. Histological examination revealed 3 with glioblastoma multiforme, 3 tuberculomas and 1 each with spongioneuroblastoma, meningioma, adenoma of the pituitary (chromophobe) and 1 unclassified tumor. Three case histories are briefly recorded. The authors note that in the aged, the clinical history of tumor may be of short duration and the number of classical symptoms small or entirely lacking.—M. E. H.

Lesions of the Aqueduct of Sylvius. WILSON. H., and LUTZ, W. G. [Yale Univ. Sch. of Med., New Haven, Conn.] *Radiology*, 46:132-138. 1946.

The roentgen demonstration of the occlusion of the aqueduct of Sylvius is often difficult. Where conventional views fail to demonstrate the aqueduct, laminography may give clear visualization. Localization of the site of obstruction is very important. A group of cases of obstructive hydrocephalus was studied by the authors, 11 of which were produced by neoplastic stenosis of the aqueduct and 3 were due to non-neoplastic stenosis. A new roentgen sign for differentiation of supratentorial and infratentorial lesions is described. This is based on the measured angle included between a line drawn along the base of the anterior fossa and a second line drawn from the anterior clinoid process to the suprapineal recess. The theories of pathogenesis of non-neoplastic stenosis of the aqueduct are discussed.—R. E. S.

Schwannoma of the Pharynx with Paralysis of the Vocal Cord. TURCHIK, F. [St. Vincent's Hosp., Bridgeport, Conn.] *Arch. Otolaryng.*, 44:568-573. 1946.

A description is presented of a Schwannoma located in the right vagus nerve at its exit from the jugular foramen. Among the symptoms observed were unilateral paralysis of the vocal cord, Horner's syndrome, weakening of the trapezius muscle and progressive involvement of the trunk, pharyngeal and palatal musculature.—C. R. N.

Neurinoma of the Facial Nerve. KETTEL, K. [Hilleröd, Denmark] *Arch. Otolaryng.*, 44:253-260. 1946.

This paper adds another facial neurinoma to the 16 previously reported in the literature. It was located in the pars descendens of the facial nerve of a 32 year old woman. The question is whether it is correct to regard the facial neurinomas as examples of neurinomas originating from an unmixed motor cranial nerve. As several authors have maintained that the seventh nerve contains

some sensory fibers, and as neurinomas practically always arise from sensory nerves or from sensory roots of mixed nerves, this tumor might possibly have arisen from Schwann cells of sensory fibers. Also suggestive of this explanation is the fact that the patient suffered severe facial pain for several years. The literature is reviewed. —C. R. N.

BREAST

The Value of Orchiectomy in the Treatment of Carcinoma of the Male Breast. LECCUTIA, T. [Harper Hosp., Detroit, Mich.] *Radiology*, 46:441-447. 1946.

Two cases of advanced carcinoma of the male breast were submitted to orchiectomy. In the first case in which there was extensive osseous metastases there was marked improvement and the patient was well 2 years after operation. In the second case there was local recurrence and liver metastasis. A temporary improvement was obtained but within a few months the patient succumbed to widespread visceral disease. These cases suggest that osseous metastases can be brought under control by castration whereas local recurrence and visceral metastases are influenced little or not at all. This conforms with present experiences with castration in mammary carcinoma of the female.   R. E. S.

Surgery of the Breast: A Review. BURTON, J. A. G. [Univ. of Glasgow, Glasgow, Scotland] *Glasgow M. J.*, 27:345-354. 1946.

This review deals with malformations, injuries, inflammatory conditions, and tumors of the breast. —E. L. K.

Paget's Disease of the Nipple. With Special Reference to the changes in the Ducts. INGLIS, K. [Univ. of Sydney, Sidney, N. S. W., Australia] *Am. J. Path.*, 22:1-33. 1946.

Two cases are reported and an account is given of the changes that occur in Paget's disease. Evidence is submitted in support of the opinion that the epidermal lesion of the nipple precedes the infiltrating cancer in the underlying breast and is linked to this infiltrating cancer by spreading down one or more ducts by the intraepithelial route.—J. G. K.

Papillomata of the Breast. WAKELEY, C. [King's Coll. Hosp., London, England] *Lancet*, 1-62. 1947.

Intracystic papilloma was the cause of a blood-stained discharge from the nipple in 62 of 119 patients treated by the author. Local excision of the tumor and not local excision of the breast is the best treatment. Follow-up records of 45 of the 62 cases show that in not one case has there been any malignant disease after local excision. X-ray and radium therapy are useless. The age incidence is from 20 to 60 years with a maximum between 40 and 45 years. Most of the tumors in the author's series were near the nipple, but 5% were at the periphery of the breast and one was in the axillary portion. --E. L. K.

Diagnosis and Treatment of Carcinoma of the Female Breast. OBERHELMAN, H. A. [Loyola Univ. Sch. of Med., Chicago, Ill.] *S. Clin. North America*, 26:116-129. 1946.

In the diagnosis and treatment of breast cancer, the value of early recognition and early surgery is duly stressed. The author believes that every breast "lump" should uncompromisingly be submitted to biopsy. The diagnostic procedures of aspiration, transillumination and radiography should be looked upon as having limited value as accurate diagnostic procedure, and they are not practical for routine breast examinations.

The employment of methods of treatment, such as castration and sex hormone therapy, should be regarded as palliative and not curative. The method of choice in combating cancer of the breast is meticulous radical or modified surgery, combined with irradiation therapy.—J. L. M.

FEMALE GENITAL TRACT

Dysgerminoma of the Ovary with Widespread Metastases. PENDERGRASS, E. P., and SELMAN, J. [Univ. of Pennsylvania Hosp., Philadelphia, Pa.] *Radiology*, 46:377-379. 1946.

A case is reported of dysgerminoma of the ovaries in a child whose first symptom was an orbital tumor.—R. E. S.

[Dermoid Cyst of Ovary, with Torsion and Infarction.] Case Records of the Massachusetts Gen. Hosp. Case 32362. *New England J. Med.*, 235:340-341. 1946.

A report of a case.—M. H. P.

Carcinoma of the Fundus Uteri. CROSSEN, J. R. [St. Louis, Mo.] *South. M. J.*, 39:445-452. 1946.

The problem and evaluation of present therapeutic methods is discussed, and the use of a flexible wire distributor for placing radium preoperatively in the uterine cavity is described. —W. A. B.

Inspissated Blood and The Growth of Fibromatous Uterine Tumors. MARSHALL, W., HOLLOWAY, A. L., IRBY, I. L., and PEACOCK, C. [Mobile, Ala.] *Am. J. Surg.*, 72: 57-62. 1946.

The theory of an active chemotropism operating in the process of fibromatosis, as advanced in a previous article by Marshall, is that serum extravasated in tissue draws fibroblasts into that area. This concept is suggested as explaining the etiology of uterine fibroids. A preliminary experiment in which an attempt was made to inject whole blood into the uterine horn of a 6 months old female puppy, failed to demonstrate any changes in the uterine horn 2 months after the injection.—W. A. B.

[Endometriosis.] Case Records of the Massachusetts Gen. Hosp. Case 32482. *New England J. Med.*, 235: 801-803. 1946.

This is the report of a case in which endometriosis involved the fragments of 2 ovaries that remained after the removal of an ovarian cyst, the posterior surface of the uterus, and probably the wall of the sigmoid.—M. H. P.

Endometriosis and Adenomyosis. YIN, Y. C. [National Central M. S., Chengtu, China] *West J. Surg.*, 54:490-493. 1946.

The author differentiates between adenomyosis (intra-uterine aberrant endometrial proliferations) and endome-

triosis (the extrauterine group irrespective of location). For endometriosis the age ranged from 19 to 32 and for adenomyosis from 30 to 45 years among a study of 40 cases. The 2 conditions might actually be treated as 2 separate diseases. It is suggested that they may be of different origins although both conditions may be activated as a result of excessive hormonal secretion of the ovaries. The fetal origin of adenomyomas is suggested. —M. E. H.

Traitement des endométriosis par l'hormone mâle. [The Treatment of Endometrioma by Male Hormone.] MOULONGUET, P. [Paris, France] *Presse méd.*, 52:194-195. 1944.

Report of 7 cases of enometriosis successfully treated by prolonged administration of testosterone.—C. A.

Endometriosis. Two Hundred Cases Considered from the Viewpoint of the Practitioner. FALLON, J., BROSNAN, J. T., and MORAN, W. G. [Fallon Clin., Worcester, Mass.] *New England J. Med.*, 235:669-673. 1946.

The triple action of endometriosis (as neoplasm, chemical irritant, and presumptive hormone manufacturer) makes almost any symptom possible. However, a basic syndrome has been identified, such as cumulatively increasing pain at the time of the menstrual period occurring after about 5 years of menstruation without pregnancy. Endometriotic nodules are pathognomonic but often not large enough to be felt. Because the disease can be seen long before it can be palpated, surgical exploration is desirable so that lesions can be excised before this becomes mechanically impossible. Of the 200 patients described, about half were treated by extirpation of lesions, and the other half by castration. A few young girls with extensive lesions were treated by removal of all lesions possible, plus temporary castration by a small dose of radium. Some castrated patients had recurrences as a result of stilbestrol administration.—M. H. P.

The Problem of Secondary Infection in Carcinoma of the Cervix. GARCIA, M., and SCHLOSSER, J. V. [Charity Hosp., and Tulane Univ., New Orleans, La.] *Radiology*, 46:448-457. 1946.

A group of 449 cases of carcinoma of the cervix treated at Charity Hospital is analyzed from the standpoint of infection. Thirty-one per cent of them showed febrile reactions that were considered significant. The type of infection, its relation to treatment and stage of the lesion, and age and color of the patient as well as the 3 year end results, and the influence of completeness of therapy, are analyzed and charted. In recent cases, penicillin appears to be a potent weapon in combating infection.—R. E. S.

Indications and Limitations of Transvaginal Roentgen Therapy for Cancer of the Cervix. ERSKINE, A. W. [Cedar Rapids, Iowa] *Radiology*, 46:458-459. 1946.

The transvaginal method of roentgen treatment of cancer of the cervix is considered to be the most efficient method of destroying the primary tumor. The author has previously reported his method which is that of re-

tracting the vaginal walls. A dose of 6,000 r can be delivered to the tissues at a depth of 3 cm. without producing necrosis of the surface of the cervix. Either 200 kv. or 135 kv. therapy apparatus can be used and adapted to the method. Contraindications to this type of treatment are vaginal atresia, occasionally vaginismus and narrowness of the vagina. Pelvic infection may postpone such treatment and it is questionable if apparently hopeless cases should be subjected to this procedure.—R. E. S.

The Treatment of Carcinoma of the Cervix. DEL REGATO, J. A. [Ellis Fischel State Cancer Hosp., Columbia, Mo.] *Radiology*, 46:579-5882. 1946.

In a general summary of the treatment of carcinoma of the cervix the author discusses the treatments of choice and survival rates for the various stages of the disease. In Stage I, which comprises only 10% of all cancers of the cervix, either thorough internal and external radiation or surgery now offers good survival rate. In Stage II the survival rate falls rapidly but adequate internal and external radiation is the method of choice. A combination of radiation and surgery in this stage does not seem justified for survival rate is not appreciably increased. In Stage III thorough external irradiation is the most important single factor in treatment. In Stage IV a few cases will be salvaged by careful and thorough external radiation.—R. E. S.

MALE GENITAL TRACT

Tumor of the Testicle: Analysis of One Hundred Cases: A Preliminary Report. LOWRY, E. C., BEARD, D. E., HEWIT, L. W., and BARNER, J. L. [M. C., A. U. S.] *J. Urol.*, 55:373-384. 1946.

One hundred cases of testicular tumor in an army general hospital are reviewed. The follow up period varies from a few weeks to 3 years. Testicular tumors represented about 70% of all genitourinary neoplasms and 7.86% of all malignant tumors admitted to this hospital. A classification is presented and the pathology summarized. Painless swelling of the testicle was the most common symptom. Diagnosis was usually correctly made on the basis of physical examination. Hormone assays of the urine were not found to be of value. Treatment was by high inguinal orchiectomy followed by deep x-ray therapy. Of 100 cases 68 are well without evidence of disease. X-ray therapy was felt to have little if any curative value in cases where metastases had already occurred but was always given for possible palliation.—W. F. W.

Lymphosarcoma of the Testicle; Report of a Case. MATHÉ, C. P. [Southern Pacific Gen. Hosp., San Francisco, Calif.] *J. Urol.* 55:530-541. 1946.

A case of lymphosarcoma of the testicle in a 63 year old male is reported. Careful examination failed to reveal any other primary site or metastasis.—W. F. W.

Hemangioma of the Testes in an Infant. ROSENTHAL, A. A. [Polyclinic Hosp., and Med. Sch., New York, N. Y.] *J. Urol.*, 55:542-544. 1946.

A case of hemangioma of the testis in an infant of 3

months is presented. Orchiectomy was necessary.—W. F. W.

Gonocytoma. Homologous Ovarian and Testicular Tumors. I. With Discussion of "Mesonephroma ovarii" (Schiller: Am. J. Cancer 1939). TELUM, G. [Univ. Inst. of Path. Anat., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, 23:242-251. 1946.

This is a report on the congruity between ovarian tumors (Schiller's mesonephroma) and adenopapilliferous, solid or cystic tumor of the testis. The gonocytoma, here defined, is "the intermediate form of the semin-oma series" and is characterized histologically by its origin from the germ cells in the testis or from homologous remnants of the medullary cords in the ovary. Case reports of testicular tumors are given, one of which exhibited many characteristics of an ovarian tumor. In contrast to the tumor described by Schiller, the growth here presented must be regarded as a true mesonephroma. —E. B. B.

Arrhenoblastoma—Androblastoma. Homologous Ovarian and Testicular Tumors. II. Including the So-Called "Luteomas" and "Adrenal Tumors" of the Ovary and the Interstitial Cell Tumors of the Testis. TELUM, G. [Univ. Inst. of Path. Anat., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, 28:252-263. 1946.

A feminizing tumor of the testis is decribed, which exhibits complete morphological congruity with the virilizing arrhenoblastoma of the ovary described by Meyer. The patient, a man aged 53, had had a testicular tumor for 30 years. For 3 years he had been impotent and during the last year gynecomastia had developed. This condition subsided after removal of the tumor. Histological examination of the growth revealed considerable lipoid content. The author concludes that testicular androblastomas should be considered as hormone-producing tumors showing all known stages of differentiation from the arrhenoblastoma. Tumors that are virilizing in women may also be feminizing in men, a fact that corresponds to previous findings concerning tumors of the adrenal cortex.—E. B. B.

A Study of the Effect of Stilbestrol Therapy on the Size of the Benignly Hypertrophied Prostate Gland. PEARSON, E. L., [Salem, Mass.] *J. Urol.*, 55:73-78. 1946.

By the use of a Foley catheter in the bladder and a balloon in the rectum, both bags distended with sodium iodide, the effect of stilbestrol therapy on the anterior-posterior diameter of the benignly hypertrophied prostate was studied from appropriate x-rays. In 10 of 13 cases this diameter was reduced 5 to 10 mm. In 3 cases there was no appreciable change in size. Five cases showed a decrease of 10 mm. or more. The average reduction for all 13 cases was 7 mm., representing an average reduction of 12%. There was little or no apparent reduction in the vertical diameter. These objective observations appear much more significant than any attempt at evaluation of clinical improvement or rectal findings. The author concludes that the general use of stilbestrol therapy is not warranted in benign prostatic hypertrophy.—W. F. W.

Primary Sarcoma of Prostate Gland in a Soldier. NEWMAN, H. R. [AAF Regional Station Hosp., Hunter Field, Ga.] *J. Urol.*, 55:295-297. 1946.

Report of a case.—W. F. W.

Questionable Cancer of the Prostate Gland: Clinical Versus Routine Pathologic Evidence. LAZARUS, J. A. [New York, N. Y.] *J. Urol.*, 55:618-625. 1946.

The author discusses the problem of the proper course of action where there are clinical signs of prostatic cancer, but where pathological examination does not confirm the clinical diagnosis. Discrepancies between clinical and pathologic data in certain cases of prostatic cancer may depend upon: (1) failure to cut sections from an adequate number of tissue blocks, and (2) failure of the pathologist to receive tissue from the part of the prostate harboring the carcinoma. The exclusion of the so-called "false capsule" of the gland in prostatectomy is the principal cause of failure to remove the carcinomatous area. This is particularly true in suprapubic and transurethral prostatectomies. The author advises castration with or without estrogen therapy in case of clinical prostatic cancer even though the pathologist may be unable to substantiate the diagnosis.—W. F. W.

Primary Carcinoma of the Prostate of Twelve Years Duration; Case Report. FLYNN, J. E. [Army and Navy General Hosp., Hot Springs, Ark.] *J. Urol.*, 55:626-630. 1946.

A 71 year old man who had a suprapubic prostatectomy for urinary obstruction was found to have an occult adenocarcinoma in the excised gland. Nine years later he developed pain in the bone and was found to have skeletal metastases. He survived 3 more years after castration and estrogen therapy.—W. F. W.

Liver Changes and Other Effects of Diethylstilbestrol During Treatment of the Prostate Gland Cancer. WATTENBERG, C. A. [Washington Univ. Sch. of Med., and Barnes Hosp., St. Louis, Mo.] *J. Urol.*, 55:631-640. 1946

A case of toxic hepatitis, apparently resulting from the use of large doses of diethylstilbestrol for the control of a prostatic cancer, is described. Changes in the breast, urethra, verumontanum, testes and prostatic carcinoma, due to diethylstilbestrol, are discussed. —W. F. W.

Carcinoma of Prostate Treated with Oestrogens. FERGUSSON, J. D. [Central Middlesex County Hosp., Middlesex, England] *Lancet*, 2:551-556. 1946.

The author considers that most carcinomas of the prostate originate in that portion of the gland lying below and behind the verumontanum. Biopsies were carried out at intervals of from 10 days to 23 months and in a few cases over periods up to 3 years. A minimum of 1.5 to 2.0 gm. of tissue was removed on each occasion. Stilbestrol or dienestrol was given in doses of from 2 to 15 mgm. daily. The specimens were stained for acid phosphates by the method of Gomori. A few normal adult prostates do not stain but in most, the enzyme is most

abundant in the posterior portion. Carcinomatous prostates generally show a consistently large amount of enzyme throughout. In 4 cases, chemical estimations showed a fall in acid phosphatase content of the prostate under treatment. There is no simple relation between the concentration of acid phosphatase in the prostate and in the serum. In every case where the primary growth showed a positive reaction, the metastatic node was also positive. Lymph glands from many other conditions were examined and acid phosphatase was rarely found. Hence it is assumed that the presence of a considerable amount of acid phosphatase in lymph gland bearing metastases suggests strongly a primary growth in the prostate.

The author has given 40 to 50 mgm. of dienestrol daily to his patients without adverse results other than vomiting. Vertigo may occur under estrogen therapy and should be considered a warning. There may be almost complete regression of the primary growth. The survival period is prolonged by estrogen treatment. A graph is given showing the length of life in 23 cases treated with estrogen and 27 cases treated in other ways. Approximately one-half the deaths of patients receiving estrogen therapy were caused by delayed, and often sudden, reactivation of the growth.—E. L. K.

The Role of Bilateral Orchiectomy in the Treatment of Carcinoma of the Prostate Gland. A Report of 82 Cases. Scott, W. W., and Benjamin, J. A. [Sch. of Med. and Dent., Univ. of Rochester, Rochester, N. Y.] *Bull. New York Acad. Med.*, 21:307-332. 1945.

A survey of 82 patients treated for carcinoma of the prostate by bilateral orchiectomy revealed a more rapid and satisfactory amelioration of metastatic symptoms than had been afforded by previous methods. However, urinary symptoms did not show as much benefit, and within 36 months most of the patients were either dead or had lost their initial gains. Because of the discouraging end results, the authors recommend a follow-up radical perineal prostatectomy in selected cases in which the response to therapy is such that there is a reasonable chance for the removal of all or almost all of the primary lesion. They conclude that: "As yet there is no substitute for radical perineal prostatectomy for cancer of the prostate gland where the patient's condition and size and location of the lesions at the time of the first examination permits its complete removal."—M. H. P.

The Estimation of Prostatic Phosphatase in Serum and its Use in the Diagnosis of Prostatic Carcinoma. Herbert, F. K. [Med. Sch., King's College; and Royal Victoria Infirmary, Newcastle-upon-Tyne, England] *Quart. J. Med.*, 39:221-241. 1946.

Estimations of serum acid phosphatase were made on 87 patients with prostatic carcinoma, 95 of prostatic hypertrophy, and 153 of a wide variety of diseases. Abnormally raised titers (5-281 units) were found in most of the prostatic carcinoma cases with metastases in bone and also in a few cases without demonstrable bone metastases. A few patients with non-prostatic disease showed titers above normal.

In order to assist diagnosis in cases showing only a slight rise in phosphatase, methods of inactivating prostatic phosphatase were studied. Prostatic phosphatase can be inactivated (a) by incubation of serum at 37° C. for 1 hour, or (b) by treatment of the serum with 2/5 volume of ethanol for a half hour at room temperature. Inactivation by alcohol treatment is recommended as a specific test for prostatic acid phosphatase. It gives an approximate quantitative measure of the prostatic fraction of the acid phosphatase and clearly distinguishes between raised titers due to prostatic phosphatase and raised titers of other origin. By the use of this test prostatic phosphatase can sometimes be detected even when the total titer of the serum is not raised.—F. L. W.

Primary Malignant Tumors of the Retrovesical Region with Special Reference to Malignant Tumors of the Seminal Vesicles; Report of a Case of Retrovesical Sarcoma. Lazarus, J. A., *J. Urol.*, 55:190-205. 1946.

Only 3 cases of primary sarcoma (including 1 new case presented by the author) and 20 cases of carcinoma.were found in a review of the literature of primary malignant tumors of the retrovesical region involving the seminal vesicles. Of the 20 carcinoma cases, the author feels that only 7 can be reasonably listed as authentic since the prostate was involved in the malignant process in 13, introducing the possibility of a primary prostatic cancer in the latter group. Brief abstracts of the recorded cases are presented. This disease is most prevalent among old men, and the left vesicle is involved slightly more frequently than the right (57.1% vs. 42.8%). Urinary symptoms ranging from increased frequency to acute urinary retention were present in about 50% of the cases where the symptomatology was noted in the protocols. In 12 of the 20 cases, statements regarding metastases were noted and of this number 66.6% occurred in the regional lymph nodes, 41% in the liver and 33.3% in the lung. The mortality in this group of cases was about 85%. —W. F. W.

Cancerous and Precancerous Lesions of the Penis: A Clinical and Pathological Study Based on Twenty-three Cases. Melicow, M. M., and Ganem, E. J. [Coll. of Physicians and Surgeons, Columbia Univ., New York, N. Y.] *J. Urol.*, 55:486-514. 1946.

Nineteen cases of primary penile cancer constitute the basis for this report. The clinical and pathological features of cancer of the penis are discussed with respect to incidence, etiology, pathogenesis, duration of the disease, pathology, symptomatology, metastases, diagnosis, treatment, and association with other disease. Four instances of secondary penile cancer are also presented and the literature on that subject reviewed. Prophylactic circumcision to reduce the incidence of penile cancer is recommended as a routine for all healthy male babies. Routine inguinal lymph node dissection in all cases of penile cancer is advised except in patients with widespread metastases and in patients who are unable to tolerate extensive surgery for other reasons. Metastases may develop by penetration of the dorsal vein of the penis as well as by lymphatic channels. Precancerous lesions of the penis are discussed and their pathological histology demonstrated. —W. F. W.

Degenerative Changes Induced in Tumor Cells by *Serratia marcescens* Polysaccharide*

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Preliminary reports of these investigations by Diller and Shear (9) and Diller (8) indicated in a general way the degenerative responses of tumor cells to a polysaccharide derived by Shear and his associates (18) from *Serratia marcescens* culture filtrate. The present paper comprises the detailed account of these cellular changes.

The use of bacterial toxins in tumor therapy was reviewed by Shear (15, 17) and more recently the application of bacterial agents in the form of Coley's "mixed toxin" to human neoplasms was surveyed by Nauts, Swift and Coley (13). However, the literature reveals no more than superficial notice of the effects of such agents on tumor cells. Apitz (4) in 1933 noted that there is an edematous appearance of tumor cells following toxin treatment, which may be separate from the anoxia caused by hemorrhage; and Andervont (3) in 1936 reported that treatment with meningococcus and *B. coli* filtrates causes tumor cells to swell prior to hemorrhage "indicating the bacterial products may have some direct effect on tumor cells."

This hypothesis is supported by a microscopic study, made over a 2 year period in our laboratory[1] of the effects produced by Shear's polysaccharide on the tumors of rats, hundreds of mice, and some hospital patients.

MATERIAL AND METHODS

Most of the studies were carried out on transplantable mouse sarcoma 37, supplemented by spontaneously occurring rat and mouse carcinomas and by human biopsy material from 16 sarcoma patients.

Small pieces of each tumor were routinely smeared for rapid examination in acetic-orcein, while the remainder was preserved in a modified Bouin's fixative (1.5 gm. of urea added to the standard formula) so that when supplementary data were needed, paraffin sections could be prepared for orientation studies and pathologic diagnosis. For demonstration of cytoplasmic components and archoplasmic structures, the coverslip smear technic described by Diller (7) was used.

The hosts for transplanted tumors were Carworth Farms albino mice (usually males) approximately 3 months of age. No difference could be noted between males and females with regard to polysaccharide response. Animals with well-established 6 to 10 day tumors which had not perforated the skin were chosen for treatment. Tumors open to the surface tend to be polysaccharide resistant, as noted by Shear and his associates (18), owing probably to the presence of infectious organisms that enhance immunity. Actively proliferating 7 day tumors arising from fragments implanted with a No. 12 trochar in a dorsal position are flattened ovals at least 7 to 10 mm. in their longest dimension, and tumors that had not attained approximately this size were discarded.

The standard mouse dose of polysaccharide employed for the experiments was 0.01 mgm. in a volume of 0.1 cc. of sterile saline, injected intraperitoneally. Other amounts employed are mentioned specifically. Intravenous injection in mice did not alter the course of cytologic response, and the simpler intraperitoneal method was therefore used. The material is highly toxic, and the amount stated usually caused death within 24 hours for 2 to 3 out of every 10 animals treated. Frequently death occurred at 4 to 5 hours, before the maximum cellular response could be realized. At higher dose levels, animals sometimes continued to suc-

cumb as late as 48 hours after administration of the polysaccharide, but when death followed a 0.01 mgm. injection, it occurred most frequently during the first night after treatment.

RESULTS

Effects on Tumors in Animals

Hemorrhage production in sarcoma 37 following polysaccharide administration has been described by Shear (17) and within the limits of individual variation, similar phenomena appeared during our experiments. Cytologically, individual tumors are not equally responsive, a fact which may be dependent upon the number of dividing cells present and the growth pattern of the tumor, as will be discussed subsequently. Microscopic observations indicate that cellular damage is produced in tumors showing little or no hemorrhage, as well as in those in which hemorrhage is extensive, but many tumors are only partially affected. However, no tumor thus far examined failed to respond to a greater or lesser degree by cellular degeneration, whether or not there was gross hemorrhage. In general, the areas of the tumor which were in closest proximity to the blood supply were first and most drastically affected.

For our cell studies, tumors were examined postinjection at hourly intervals, beginning with ½ hour after treatment. At 1½ to 2 hours the first cellular responses appeared in sarcoma 37, whether or not there was macroscopic hemorrhage. Prophase nuclei were primarily affected and reacted by forming surface blisters, or blebs, which were sometimes minute, at other times were more extensive, and in extreme cases there were multiple blebs. (Figs. 2, 3, and 27). In the last case, the entire nucleus appeared to have swollen, and at times pseudopodial processes (Fig. 3) were thrust out.

From 2 to 2½ hours, fewer nuclei with blistered membranes were encountered. Instead, many nuclei were shrunken, as though by withdrawal of fluid. As revealed in paraffin preparations, there was a corresponding shrinkage of the cytosome, which resulted in the disruption of the tumor tissue through separation of the cells into discrete entities. Resting nuclei were in most instances morphologically unchanged; occasionally, however, they were enlarged through apparent imbibition of fluid, but no blistering of their membranes was observed.

Correspondingly, those nuclei which were in metaphase at 2 hours after treatment showed considerable damage (Fig. 9). A series of changes appeared to have taken place between 2 and 3 hours which involved various aberrations in nuclear structures. Some were slight, *e.g.*, the displacement of individual chromosomes with respect to the metaphase plate; others showed drastic disorganization such as that in Fig. 10. Furthermore, there was a loss of stainability, which caused the center of the chromosome to appear hollow and vesiculate, although the outer rim was heavily stained. Sometimes these transparent bodies had become confluent and produced enlarged hyaline structures like those of Fig. 5. At other times, coagulation involved all the chromatin, as in Fig. 7.

Nuclei that arrived at the anaphase before this period were apparently successful in completing division, for there was no evidence of anaphase or telophase degeneration; therefore, polysaccharide did not appear to suppress division through any action on the spindle, as is the case with colchicine. At 2 to 3 hours after treatment, cell division was still uninhibited; though a considerable number of prophase nuclei showed surface modifications, and numerous metaphases were disrupted.

Preparations made between 3 and 3½ hours postinjection indicated a more advanced stage of degeneration involving pycnosis of large numbers of nuclei and considerable shrinkage of many others. These nuclei appeared more concentrated with decreasing size and assumed a bean or kidney

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DESCRIPTION OF FIGURES 1 TO 14

Camera lucida drawings from acetic orcein smears (unless otherwise indicated) of sarcoma 37 treated with 0.01 mgm. of *S. marcescens* polysaccharide. Mag. × 2,500 (approx.).

Fig. 1.—Untreated prophase nucleus.

Fig. 2.—Prophase nucleus of teraploid cell (4 nucleoli) 2 hours after treatment. Note blistering of nuclear membrane.

Fig. 3.—Prophase nucleus, 2 hours after treatment; note shrinkage of nucleus and production of pseudopodial process.

Fig. 4.—Prophase nucleus, 3 hours after treatment.

Figs. 5, 6, and 7.—Metaphase nuclei, 2 hours after treatment.

Fig. 8.—Shrunken nucleus, 3 to 4 hours after treatment.

Figs. 9 and 10.—Metaphases, 3 to 4 hours after treatment.

Fig. 11.—Extrusion of nuclear filaments, 4 hours after treatment.

Fig. 12.—Extrusion of a single longitudinally divided chromosome from metaphase clump 4 hours after treatment (paraffin preparation, Feulgen stain).

Figs. 13 and 14.—Distorted nuclei with extruded filaments, 4 to 5 hours after treatment (paraffin preparation, hemalum stain).

Figs. 1-14

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17
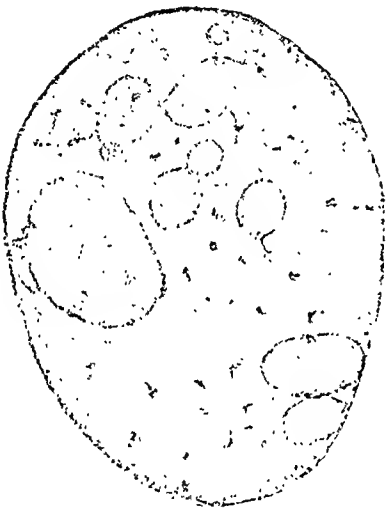
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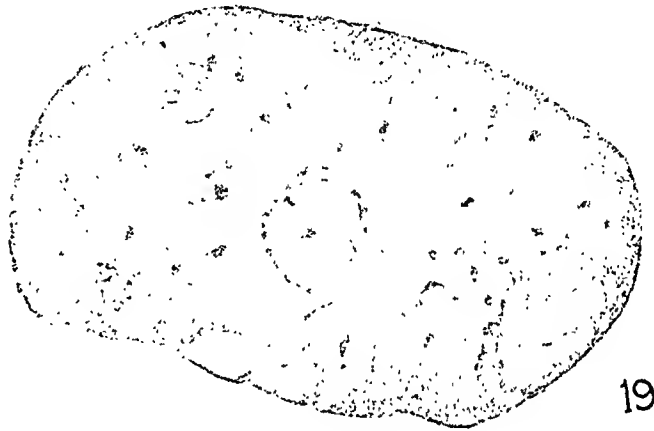
19
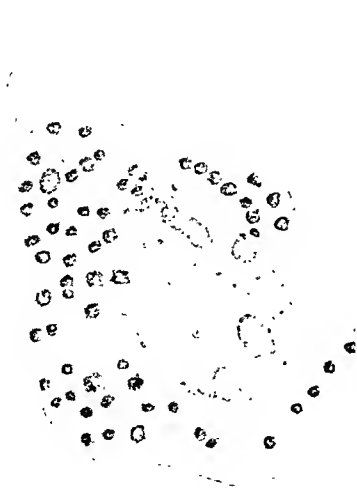
20

21

22

Figs. 15-22

shape (Figs. 4 and 8). Whether these always arose through collapse of previously swollen nuclei, it was not possible to judge. Stages indicating progressive shrinkage of nucleoli were likewise demonstrable, and these bodies ultimately came to be represented only by dark granules that took orcein stain, but no fast green. The cell body also decreased in size and was more heavily stained. Blebbed nuclei could also be found. Metaphases were practically all degenerative, e.g., Fig. 6 (see also Figs. 30 and 31), and there were no anaphase or telophase figures. Sister cells which were in the process of separation at 1½ hours apparently succeeded in completing division, but thereafter the division process usually did not go beyond the metaphase. In many tumors studied the only normal cells visible at 3 hours post treatment were resting ones. Some of the tissues showed large areas in which the orcein, staining the nucleoproteins, was dispersed throughout the cell instead of being limited to chromatic structures. Furthermore, there appeared at about this time nuclear disruptions so drastic that they must certainly be irreversible. They were the most characteristic feature of polysaccharide response.

The essential feature of this change was the extrusion from the nucleus of filamentous orcein-positive (chromatin) bodies, sometimes involving many threads and sometimes consisting of a single chromosome pair (Fig. 12). Here the chromatin material of what is apparently a degenerating metaphase figure is clumped into a formless mass for which the longitudinally double chromonemata of a single pair of chromosomes have been extruded far beyond the confines of the nucleus, and even of the cell body, to attain an extended dimension approximately 4 times that of the clumped nucleus. This figure (shown also in Fig. 32) is from a paraffin preparation subjected to the Feulgen reaction. The greatly extended thread is Feulgen-positive and still exhibits some faint traces of residual coiling. A dimly discernible body attached to the pycnotic distal granules appears to be the remnant of the nucleolus.

A similar attenuation involving remnants of prophase nuclei appears in Figs. 11 and 13. Even

more distorted nuclei filled with fairly individualized chromatin threads drawn out to a considerable length are also present (Fig. 14). These figures are better demonstrated in smears than in paraffin sections, where they are so long that they can seldom be found in one section but appear in cross section as chopped-up fragments. It is barely possible that the pressure exerted in making the smear preparation may tend to exaggerate the effect; but the same phenomenon is apparent to a lesser degree in paraffin preparations, where the tendency is in the other direction, toward shrinkage and a consequent masking of the early effects of the polysaccharide.

At 4 hours practically all metaphase nuclei were clumped (Fig. 8) whether or not there were chromatin extrusions from the central mass. By this time some of the metaphases were nothing but pycnotic spheres, sometimes perforated by vacuoles (Fig. 6). No normal metaphases, and no telophases at all, persisted. Such intact cells as survived were in resting stage, but they often appeared greatly swollen and contained huge, correspondingly swollen nucleoli. Blebbed and reniform nuclei were also present.

A different kind of degeneration of the tumor sometimes occurred about 4 hours postinjection. This involved a complete coagulation of both nucleus and cytosome (Fig. 24).

Between 4 and 5 hours when tumors grossly exhibited considerable hemorrhage, degeneration of initially affected cells neared its peak. Swollen resting nuclei, blebbed prophase nuclei, bean-shaped, shrunken nuclei, clumped metaphases and extruded filaments were all present. The shrunken prophases without extruded filaments had no semblance of ordered form and became twisted "ghosts" from which cell boundaries were lost, while the pale, muddily stained nuclear remnants lay upon an amorphous mass of degenerating cytoplasm. Individually these bodies rounded into spheres of descending sizes or faintly stained bodies of irregular contour, from which all trace of structure had disappeared. The spherical bodies apparently arose from metaphase degenerates, while twisted, folded, and flattened figures were

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DESCRIPTION OF FIGURES 15 TO 22

Camera lucida drawings; smear preparations of sarcoma 37, treated with 0.01 mgm. of *S. marcescens* polysaccharide. Mag. × 2,500 (approx.).

Figs. 15 and 16.—Degenerating telophases, newly dividing cells, 3 days after polysaccharide treatment (acetic orcein stain).

Fig. 17.—Undamaged resting nucleus, surviving polysaccharide treatment (acetic orcein).

Figs. 18 and 19.—Nuclei, enormously enlarged in resting cells, during 3 day period following polysaccharide treatment (acetic orcein).

Fig. 20.—Resting nucleus during 3 day period of mitotic inhibition following polysaccharide administration (smear, Flemming fixation, Flemming tricolor). Note "fatty degeneration" of cytoplasmic substance.

Figs. 21 and 22.—Degenerating nuclei from cells treated with polysaccharide and x-ray, 24 hours after irradiation.

remnants of nuclei in pre-metaphase stages. Degenerating blood cells were also present (Fig. 36).

The amount of destruction varied considerably, but in some tumors deterioration was so extensive that by 6 hours there was practically complete necrosis; and nothing remained but amorphous cytoplasmic debris with some nuclear fragments (Fig. 26). Recognizable cells were in an advanced stage of degeneration and seemed for the most part to be little more than distorted naked nuclei (Fig. 33).

Beyond this time (6 hours) the stages undergone by as yet undamaged cells were identical with those just described, and the result was merely an extension of the response to include greater areas of the tumor. The length of time during which degeneration can continue, and before any resistant cells are again able to resume division, is discussed in the following section.

Duration of effect.—As mentioned previously, the maximum hemorrhagic effect was reached at approximately 6 hours after injection of the polysaccharide. The tumors were usually encapsulated; and when extreme hemorrhage was induced, the neoplasm appeared on gross examination as a soft, bloody sac. It is not uncommon at this period to find such marked deterioration that at times no solid tissue is available, and little more than a bloody smear can be secured for microscopic examination. During this period the mice show lowered temperatures, accept no food or water, and remain huddled and immobile in their cages. Some casualties may occur from 4 hours onward, but survivors of the 0.01 mgm. dose usually completely recover within 24 hours. They were eager for food and could run and swing on their cages in a normal way, indicating that recovery from the toxic effects was quickly accomplished. In order to determine whether the cellular response of the tumors is correspondingly brief, any persisting residue of undestroyed tissue was studied at 24 hour intervals, up to 5 days postinjection.

For this series of studies tumors were chosen, which, because of extensive hemorrhage, showed that they had reacted. As Andervont (3) demonstrated in connection with tumor response to meningococcus and *B. coli* filtrates, tumors which are totally hemorrhagic usually form a hard mass of dried blood beneath the skin within 24 hours. In our 24 hour material, the entire tissue was sometimes involved in the hardened mass, while at other times a certain amount of translucent tissue could be secured for examination. Invariably, in such material, the only seemingly normal cells were "resting" ones (Fig. 35). In the

transition between interkinesis and the stage of active division the extreme diffusion of the chromatin substance made the distinction between "resting" and dividing nucleus difficult, if not impossible; but nuclei were arbitrarily judged to be in prophase when definite polarization of the heterochromatic proximal ends of the chromatin threads with respect to the nuclear membrane, together with resolution of definitive chromosomes, first became visible.

After 24 hours the only morphologically intact cells were those not visibly preparing for mitosis. There is some evidence that these cells, too, may respond in some way, through changes in the cytoplasmic constituents. An instance of this appears in Fig. 38. This photograph shows a single resting cell remaining undistorted amidst the debris of degenerating cells. The nuclear structures and cell membrane are intact, but the osmiophilic substance has undergone "fatty degeneration" (see also Fig. 20) and is massed in the cytoplasm as blackened spheres. Disrupted cells have extruded osmiophilic substance in the form of spherical masses, and these clumps, large and small, are scattered through the tumor 24 hours after polysaccharide administration.

Degeneration of all types described in the previous section could be discovered at 24 hours, except in cases where all the material had reached the highly attenuated condition. Blood cells were present in large quantities. Erythrocytes by this time began to disintegrate and to confuse further the already chaotic picture by disrupting into amorphous masses and fragments (Fig. 37). Alternately, their substance, which is enucleate, was extruded in filamentous conglomerates resembling in the extremity of their attenuation the drastically elongated chromatic threads already described.

At 48 hours there still were no dividing cells. Resting nuclei appeared greatly enlarged, and judging by the number of nucleoli, which were tremendously swollen and frequently coalesced (Fig. 19), many of these were heteroploid. The necrotic areas now presented an appearance of complete chaos, in which all semblance of nuclear contour had disappeared, and only pycnotic bodies and fragments remained.

The first dividing cells were noted 72 hours after injection. Some of the divisions apparently succeeded, but many were abortive. Frequently, division was halted in metaphase with resultant pycnotic malformations and condensations, but the bulk of the cells degenerated at telophase. This did not involve spindle suppression or destruction but rather the failure of sister nuclei to
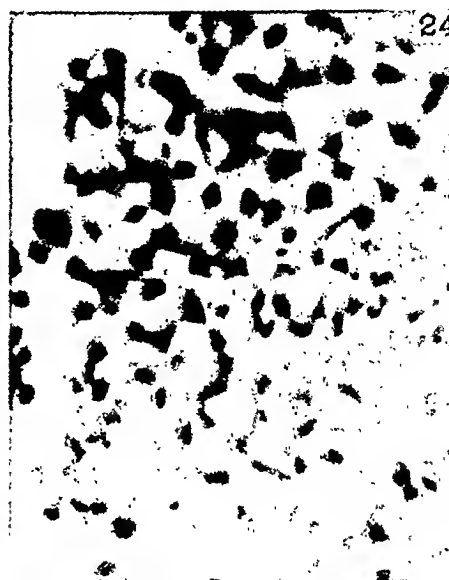
Photomicrographs from acetic orcein preparations of sarcoma 37, treated with 0.01 mgm. of polysaccharide.
FIG. 23.—Untreated control tumor. Mag. × 1,000.

FIGS. 24 and 25.—Tumor 4 to 5 hours after treatment. (Fig. 4, mag. × 720; Fig. 5, mag. × 1,000).
FIG. 26.—Same, 6 hours after treatment. Mag. × 1,000.
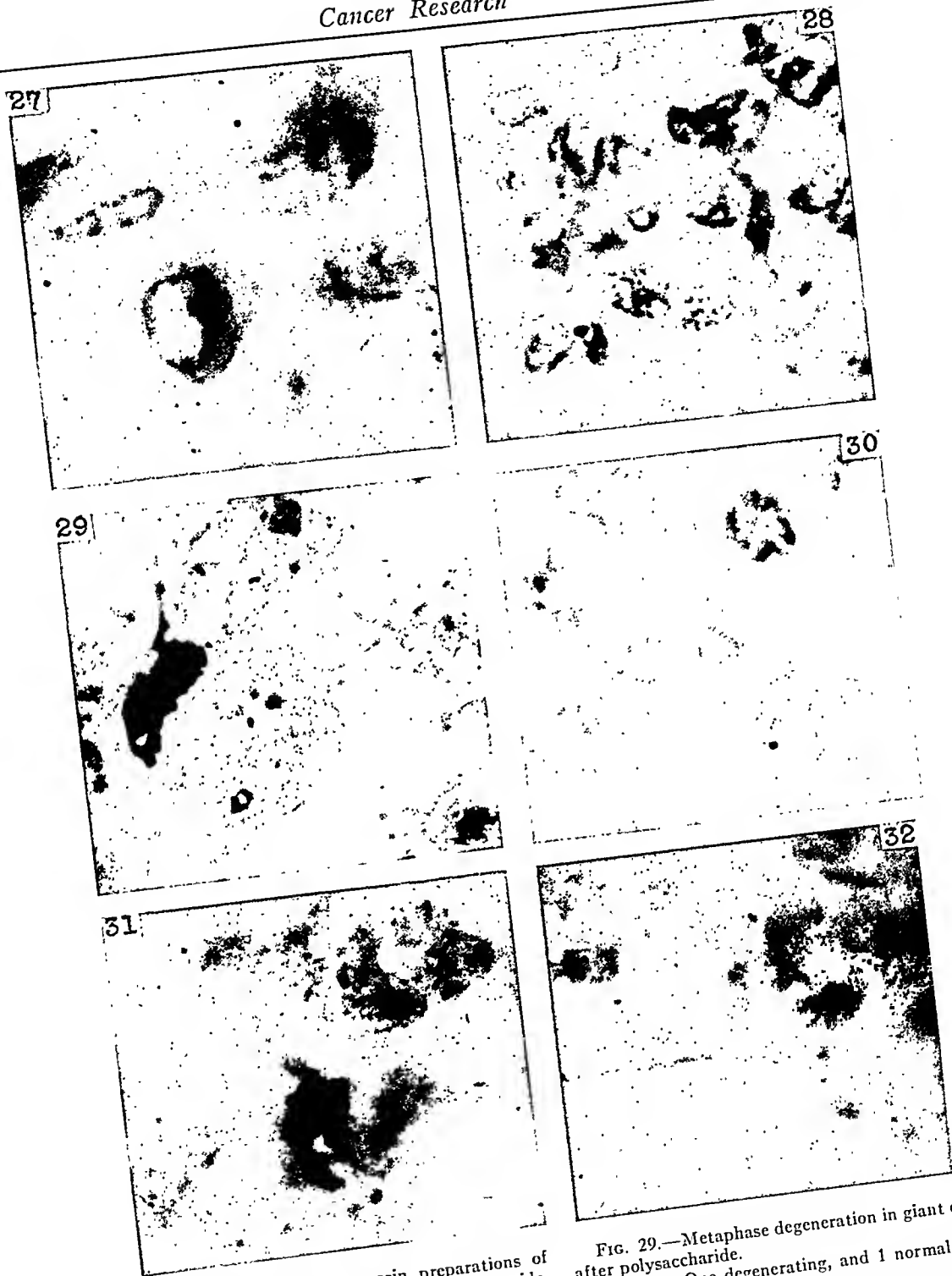
Photomicrographs from acetic orcein preparations of sarcoma 37, treated with 0.01 mgm. of polysaccharide. Mag. × 1,000.

FIG. 27.—Blebbing of nuclear membrane, 1½ hours after treatment.
FIG. 28.—Vacuolization of individual nuclei, 2 hours.

FIG. 29.—Metaphase degeneration in giant cell, 2 hours after polysaccharide.
FIG. 30.—One degenerating, and 1 normal metaphase, 2 hours after treatment.
FIG. 31.—Degenerating metaphase, 2 hours after treatment.
FIG. 32.—Same, 4 hours after treatment, showing extrusion of individual chromonemes. See also Fig. 12.

reconstitute resting cells (Fig. 16). The degenerates were frequently dumbbell-shaped figures comprised of persisting interzonal fibers or a connecting strand of nucleoplasm separating two pycnotic sister nuclei that tended to degenerate asynchronously.

If no resting cells persisted over the 3-day period the tumor was no longer viable, so far as was detected microscopically. No mitotic figures known to arise from the enormously swollen, obviously hyperploid nuclei were positively identified, but polyploid cells in every stage of division were numerous, as were also aberrant metaphases which were attempting regulation, *e.g.*, by throwing out supernumerary chromosomes or by formation of multipolar spindles. Quite possibly these are the products of the giant nuclei. Moreover, at this time it was not uncommon to find "nests" of dividing figures which might involve 6 or 8 metaphases superimposed upon one another in a single microscopic field, in a juxtaposition suggesting that they might be the products of a single giant cell.

By 5 or 6 days after treatment, tumors were again growing actively next to the body wall to an extent discernible even to the naked eye. Alternately, they were being sloughed off at the surface or shrinking down by resorption. The inhibitory effect of the bacterial substance on division processes in sarcoma 37 was, therefore, of no more than 3 days' duration. The first attempts at division were usually unsuccessful; but between 72 and 96 hours, morphologically unaltered resting nuclei were able to resume division and rapid proliferation of the tumor began.

The experiments thus far described were made on 7 to 10 day old tumors. Andervont (3) in discussing reactivity of mouse tumors to bacterial products states that the introduction of such substances "does not produce hemorrhage within skin tumors until the fourth or fifth day after inoculation, although growing tumor tissue is present as early as the third day." In order to discover whether there would be an accompanying failure of nuclear breakdown before the sixth day postimplantation, we studied a series of tumor implants treated with the usual amount of polysaccharide, at daily intervals, *i.e.*, 24, 48, 72, and 96 hours, and at 5 days after implantation. As we shall discuss later, onset of cell division in the tumor implant is probably correlated with availability of blood supply. It is evident upon gross examination that chance decides whether the implanted fragment comes to rest in close proximity to a vascular branch, or whether it must remain quiescent until capillary branching can be elicited,

a 3 day process according to Algire's studies (2). This agrees with our own cruder observations and with Andervont's statement (3) that dividing tissue is present as early as 3 days after implantation.

In our own experience, proliferation of sarcoma 37 implants is correlated with establishment of contact with the blood supply. Tumor fragments fortunately placed in juxtaposition to capillaries begin to divide at the outer edges of the implant as early as 28 hours after deposition. On the other hand, fragments not in contact with existing blood capillaries may remain as long as 3 days without blood supply until·capillary branches are established. Beyond this period they are apparently incapable of survival. In almost every case, only the part of the implant directly in contact with vascular branches enters into division; the residue degenerates.

Careful examination of many tumors reveals that when cells are dividing they will react typically to polysaccharide, regardless of the age of the implant. The same types of degeneration hitherto described (disrupted metaphases, shrunken crescents and filamentous extrusions) together with structurally normal resting nuclei, are found. Since nearly all the living tissue surrounding the young implant is in rapid division, it is not uncommon to find that 6 hours after administration of the agent to an animal bearing a 2 to 3 day implant, nothing remains except the opaque necrotic mass of implanted tissue which has not yet been absorbed, and a faint trail of blood where the capillary connections have been severed. In a few cases where the total implant had presumably become involved in division within 3 days (as indicated by the absence of any central necrotic plaque of tissue) no tumor tissue remained at the implantation site 6 hours after injection of 0.01 mgm. of polysaccharide.

Older tumors also reacted with the same type of cytologic change. Tumors were studied up to 16 days postimplantation, and although the conditions are more difficult to interpret because of the presence of large amounts of spontaneous necrosis, the expected types of damage were also encountered. Apparently a good many resting cells were dormant in these older growths, since they persisted in large numbers following polysaccharide treatment, and we could never succeed in breaking down a whole tumor but caused only a portion of it to react, as indicated by our microscopic findings and by partial sloughing macroscopically observable.

Effect of multiple or continuous treatments.— In about a fourth of the tumors examined, there was no resumption of division on the third day after

polysaccharide treatment; in the remainder, mitotic figures were plentiful. The obvious practical procedure would appear to be to reinject the host at 3 day intervals in the hope of suppressing each new mitotic wave. This experiment was tried, using the same amount and concentration of polysaccharide in each successive treatment until sloughing was induced or the animal succumbed to the tumor. Much to our disappointment, this induced no appreciable additional cell destruction; and growth, once re-established, was not noticeably impeded. Tumors treated over long periods (about 20 days) tended to become heavily encapsulated, and in only a few cases did we detect metastases. The failure of repeated doses of polysaccharide to suppress growth indicates that either the tumor or the host becomes resistant to the bacterial agent.

Since the amount of the original single injection could not be increased without causing the death of a still greater number of mice, we attempted to use polysaccharide of lower concentration in multiple doses of increasing magnitude. An initial injection of 0.001 mgm. in 0.1 cc. of fluid was administered, and thereafter the injections were increased by 0.001 mgm. daily until a total of 0.02 mgm. had been injected over a period of a week. Two series of tumors were treated simultaneously. One of these was examined microscopically on the seventh day after initial treatment, and the other series was allowed to run until the hosts either succumbed or were able to slough off or resorb the tumor. In the latter series, only 1 animal in 10 was able to rid itself of the growth. The others died within 20 to 24 days.

The tumors examined on the seventh day frequently were scabbed next to the skin and microscopically showed areas of degeneration in which no dividing tissue was present and which did not appear to arise from spontaneous necrosis. In these areas, the cytoplasm tended to be extremely viscous and coagulated. Even tumors exhibiting no gross hemorrhage but filled with clear, translucent tissue, had degenerative areas. In acetic orcein smears the affected portions were stained aberrantly, and the cytoplasmic substance, which should have been stained with fast green, was pinkish as though by diffusion of the chromatic substance from the nucleus. Where areas of dividing tissue impinged on degenerating ones, the dividing cells showed a high proportion of polyploid nuclei.

Also, we injected mice with single large doses (0.005 mgm. in 0.5 cc. of fluid) of the low concentration. At 5 to 6 hours there followed marked degeneration of all but resting nuclei, and there was total coagulation in the hemorrhagic areas. In the microscopic preparations, the tumor tissue showed clotting at the center, pycnotic rounded fragments, and heavily stained resting nuclei. At 24 hours, cell division had not been resumed in the affected areas. Nevertheless, tumors similarly treated and not removed for microscopic examination continued to grow and eventually caused the death of the host.

These experiments indicated that multiple doses of low concentration were not as effective in tumor-cell destruction as a single dose of a magnitude approximately $\frac{1}{4}$ lethal to the mouse hosts. Furthermore, multiple injections of larger magnitude did not offer much improvement over a single application in breaking down or inhibiting growth in tumor tissue.

Injection directly into the tumor.—Since our studies showed beyond much doubt that polysaccharide has a direct effect on cells apart from that following hemorrhage, it occurred to us to try to obtain cell breakdown by injecting directly into the tumor with the hope that the toxic systemic effects produced in the host could thus be obviated. Injection of 0.005 mgm. of polysaccharide into a 7 day mouse tumor resulted in the disruption and bursting of some of the cells and, in one case, in a severe coagulation of the cytoplasm. Except for this instance there was not much difference in the appearance of cells from tumors so treated and control tumors injected with the same amount of salt solution or distilled water. In no case was division suppressed for more than a few hours and growth was at best only temporarily inhibited. This agrees with observations made in another department of this Institute on tissue cultures treated directly with polysaccharide (Royle, unpublished data), wherein the response was negative.

Effect of polysaccharide on normal body tissues.—Since the hemorrhage-producing effect of *S. marcescens* polysaccharide appeared to be confined to the region of the tumor, a study was made of normal tissues of treated mice to determine whether the tissue-destructive action of the bacterial product was likewise limited to tumor cells. So far as we were able to determine, this is not the case, although tumor cells did appear to be much more sensitive than nontumor cells, and response was elicited only in those tissues undergoing division or rapid nucleoprotein synthesis. Intestinal epithelium, which appeared to be the most actively dividing tissue in the adult mouse, was also the most reactive to polysaccharide.

Following administration of polysaccharide, extensive filamentous degeneration was produced in the villi of the mouse intestine, which normally
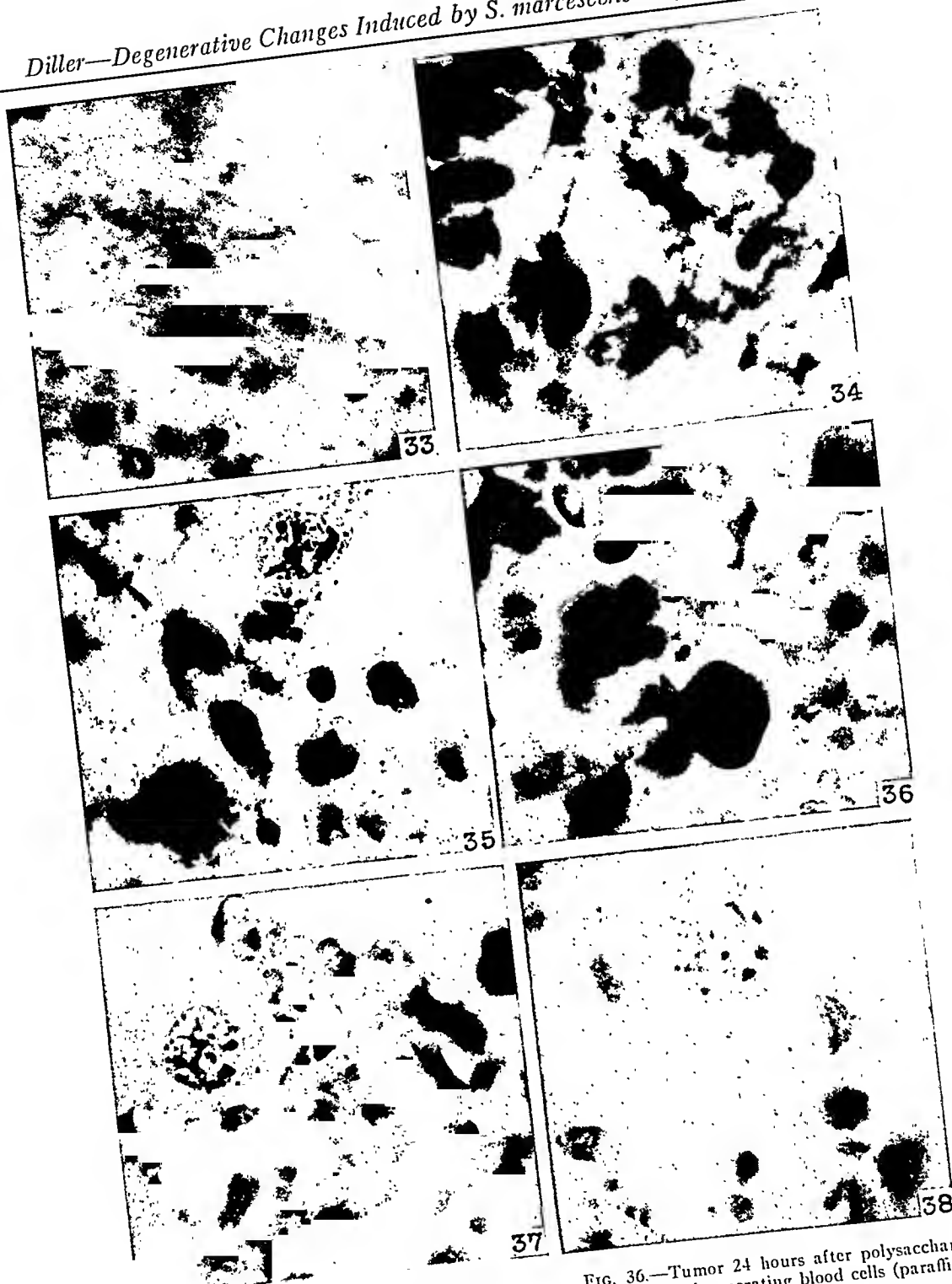
Photomicrographs from smear preparations of sarcoma 37 treated with 0.01 mgm. of polysaccharide. Mag. × 1,000.

FIG. 33.—Tumor, 6 hours after treatment (hemalum-eosin stain).

FIG. 34.—Same (acetic orcein stain).

FIG. 35.—Resting nucleus, morphologically unchanged, 24 hours after treatment. (Flemming tricolor).

FIG. 36.—Tumor 24 hours after polysaccharide treatment, showing degenerating blood cells (paraffin preparation).

FIG. 37.—Tumor 48 hours after treatment; resting nucleus and degenerating blood cells.

FIG. 38.—Three days after treatment; cytosome of resting nucleus filled with osmiophilic bodies which appear degenerative. See also Fig. 20.
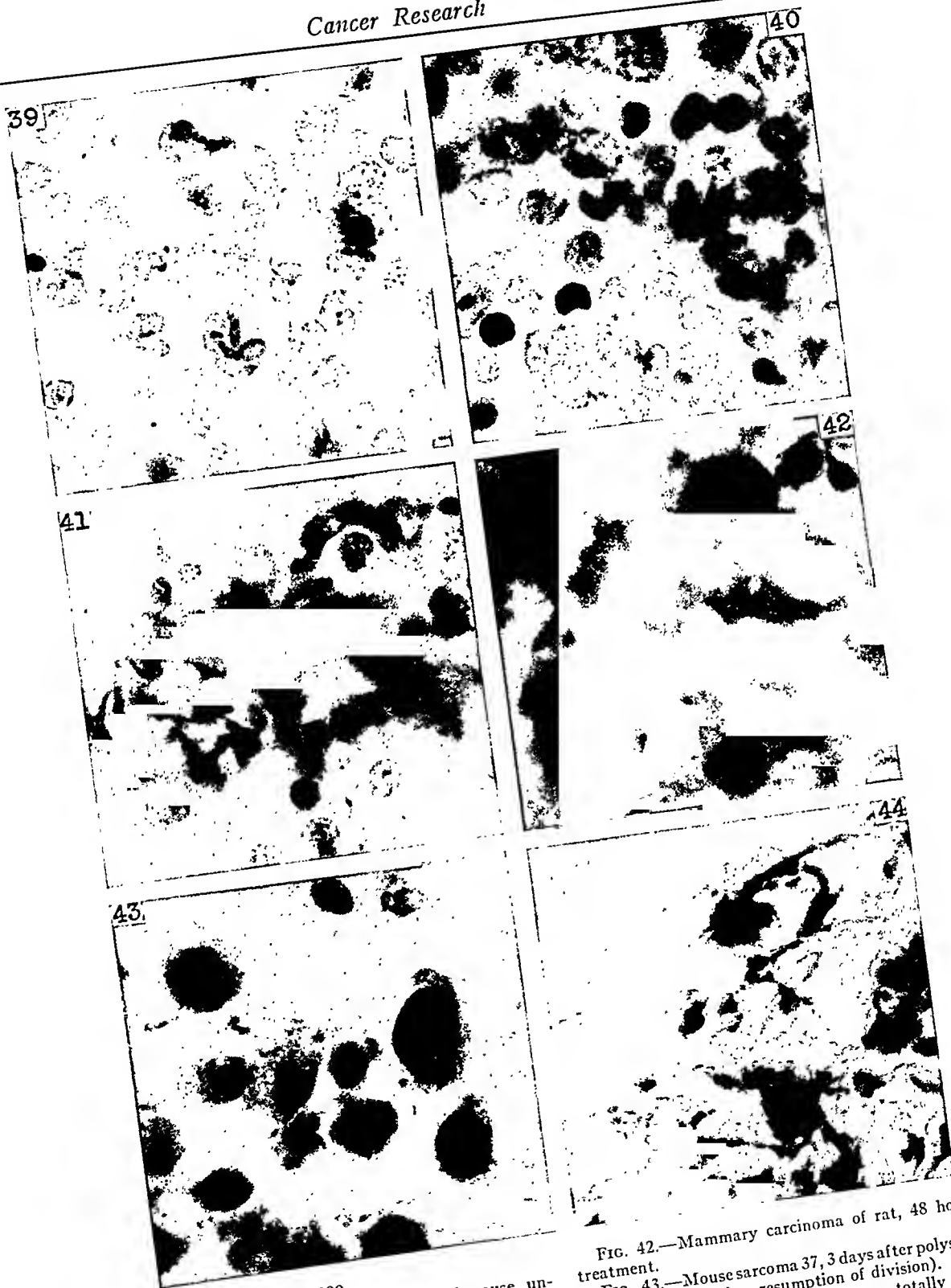
FIGS. 39 to 44 Mag. × 1,000.
FIG. 39.—Spontaneous breast carcinoma of mouse, untreated.
FIG. 40.—Same, 6 hours after polysaccharide treatment.
FIG. 41.—Same, 24 hours after treatment.
FIG. 42.—Mammary carcinoma of rat, 48 hours after treatment.
FIG. 43.—Mouse sarcoma 37, 3 days after polysaccharide treatment (just before resumption of division).
FIG. 44.—Same, specimen from a totally nonviable tumor.

contained large numbers of cells in mitosis at all times. No difference was detected in the response of intestinal epithelium of tumor-bearing mice and those having no tumors. Fortunately, the amount of polysaccharide required to produce destruction of nontumorous tissue was greater than that required for degeneration for tumor cells. For instance, the usual dose of 0.01 mgm. produced only slight breakdown in intestinal epithelial cells, and in order to secure an effect comparable to that which would occur in a tumor following administration of the same amount of polysaccharide, a concentration 10 times as great (0.1 mgm.) was required. The reaction of intestinal epithelium to this amount of polysaccharide, 6 hours after intraperitoneal administration, appears in Figs. 46 and 48 (*see* Figs. 45 and 47).

Other tissues studied were spleen, bone marrow, kidney, liver, endocrine glands, and gonadal tissue. Of these, the damage was greatest in developing blood cells of the bone marrow and in the liver. The amount required to produce extensive damage was also 10 times that used for the destruction of tumor tissue. Kidney, which has few dividing cells, was almost completely resistant. A more puzzling phenomenon was the failure of response of gonadal cells, either spermatogonia or oogonia, to polysaccharide, even when an amount fatal to half the mice was employed. (Fig. 49). When tumor-bearing males and females, treated with polysaccharide in an amount sufficient to cause the sloughing of the entire tumor, were maintained for 2 or 3 months thereafter and bred *inter se*, normal litters of offspring were produced.

The effect of polysaccharide on the adrenal and other glands will be discussed in a later paper. A decided shrinkage of cells and nuclei of the adrenal, particularly in the medullary region, was produced within 6 hours after injection of 0.01 mgm., but the effect was usually reversible at this dose level within 24 hours.

Treatment of spontaneous neoplasms.—A series of studies of the reactions of mice with primary subcutaneous tumors to hemorrhage-producing polysaccharide was made by Shear (16). From his paper the following is quoted: "Sufficient evidence . . . has been accumulated to show that the production of hemorrhage and necrosis by such agents is by no means restricted to transplanted sarcomas. While it has been found by most workers that, in general, carcinomas are more refractory than sarcomas, nevertheless, several strains of transplantable carcinomas have been reported to be responsive to the action of these bacterial products. . . . Furthermore, this hemorrhagic and necrotic effect is not confined to transplanted tumors."

The results of Shear's studies of 750 mice bearing chemically induced tumors show that, while administration of *S. marcescens* polysaccharide regularly produced hemorrhage and necrosis, some portion of the neoplasm usually escaped destruction and continued to grow progressively until the death of the host.

Spontaneous growths in rats and mice.—Cytological studies were made by us of the effect of polysaccharide on spontaneously occurring tumors that appeared in our stock colony of rats and mice, and also of a controlled series of mammary adenocarcinomas in C3H and dba mice. C3H mice with large mammary carcinomas, some of which were multiple, were treated in the same way as mice with transplanted tumors, *i.e.*, 0.01 mgm. of polysaccharide was injected intraperitoneally and the animals were killed at 6 hours. These mammary tumors were highly vascularized; and although treated tumors were scarcely more than sacs of blood at 6 hours posttreatment, we found the control tumors also to be highly hemorrhagic. Therefore the presence of a great deal of blood in the treated tumors could not in this case be taken as a criterion of polysaccharide reaction. However, microscopic comparison of control and treated materials revealed a definite cellular response which, though by no means as pronounced at 6 hours as in sarcomatous growths, was nevertheless striking (*see* Figs. 39 and 40). The extreme filamentous degeneration in sarcomas at 6 hours was lacking, but there was a rounding and pycnosis of nuclei.

A second series of mammary carcinomas in dba stock was treated with a larger amount of polysaccharide (0.02 mgm.) and the survivors were killed 24 hours after treatment. Considerable areas of the tumors were then found to be responding as in sarcoma 37, by extrusion of nuclear components (Fig. 41).

Spontaneous mammary carcinomas arising in our colony stocks of heterozygous white mice were also studied with similar results. Mammary carcinomas arising in aged albino rats maintained on butter yellow diets were also studied. Pretreatment biopsies were taken and injections of 1 cc. each of polysaccharide of low concentration (*i.e.* 0.01 mgm. of polysaccharide) were made into the rats. The animals were killed the following day and the tumors prepared by the paraffin method. Definite and typical, though not extensive, nuclear response was detectable, but the most interesting finding was that, although the capillaries penetrating into the neoplasm were still intact, the tissue immediately adjacent was highly degenerative.

EFFECTS ON TUMORS IN HUMAN BEINGS

The human material available for study was obtained from biopsies taken of 16 sarcoma patients before and after treatment at the Lankenau Hospital during the winter of 1944-45. The clinical responses of these patients and the pathological findings were reported by Holloman (10) and by Oakey (14). Four patients had previously been treated with *S. marcescens* polysaccharide at another hospital, as described by Brues and Shear (6). The biopsy material available for our studies included specimens of chondrosarcoma, fibrosarcoma, lymphosarcoma, recurrent melanoma, chronic granuloma, secondary spindle-cell sarcoma, and nodes from the neck region of a patient suffering from Hodgkin's disease. The previous finding that carcinomas do not respond as readily as do sarcomas restricted the choice of cases for study to those with sarcomatous growths.

The course of cytological response followed the pattern already exposed by our studies of animal tumors. It differed only in the relatively small amounts of tissue affected. This was to be expected because the amounts of polysaccharide administered to human beings were relatively small in proportion to body weight compared with the doses employed in treatment of mice. As the previous clinical reports describe (10,14) human beings responded with much greater intensity than did mice, as far as toxic effects were concerned, and for this reason in the preliminary trials the amounts used were minute, *i.e.*, 0.01 mgm. in a volume of 1 cc. administered intravenously. Our studies with mice had shown that in sarcoma 37 the ultimate cytological response was the same regardless of the route of injection. The cellular conditions were so similar in all the sarcomas studied that it is not necessary to present examples of every case, but the findings on 4 different types of human neoplasm are presented. Figures 51 to 54 are photographs from paraffin preparations used in pathological diagnosis and are typical of the general histological picture following polysaccharide treatment. The remainder of the figures are from acetic orcein smears prepared especially for nuclear study.

Fibrosarcoma.—This was a very large growth of long standing that involved the entire right arm of a young man. The cell conditions before treatment, as revealed by biopsy, are shown in Fig. 57. Fig. 58 was made from biopsy material excised 4 days after administration of a single dose of 0.01 mgm. of polysaccharide. Externally, the sarcoma was black and blue because of hemorrhage and the gross appearance of the interior, once hard, white and glistening, was soft and blood-filled.

Cells from tissue adjacent to these areas showed degeneration similar to that in mouse sarcomas.

Chondrosarcoma.—The patient had several metastatic tumors growing in the nose and orbital fossa and was beyond further surgical aid. She was given a total of 0.033 mgm. of polysaccharide in 3 separate applications. Two biopsies were taken, at 4 days and 6 weeks, respectively, after treatment. The first posttreatment biopsy showed a part of the tumor to be extremely hemorrhagic. Cells from this region were in the anticipated stages of degeneration (Fig. 56).

Hodgkin's disease.—Another example of polysaccharide response appears in Fig. 60 taken from a neoplasm originally diagnosed as lymphosarcoma of the axillary nodes, but which was later determined to be Hodgkin's disease. The patient was given a total of 9 cc. or 0.09 mgm. of toxin, in three equal doses over a 2 day period; 3 days after cessation of treatment the sarcomatous nodes in the supraclavicular space were dissected out. In contrast to those removed before treatment (which had been firm and of the usual color) these were cyanotic, soft and necrotic. A comparison of the two figures taken from this material (Figs. 59 and 60) shows the extreme extrusion of chromatin threads after treatment, and degeneration of cell nuclei.

Lymphosarcoma.—This neoplasm was imbedded in the groin and was so indefinitely delimited that it was inadvisable to secure biopsy material for pretreatment examination. Therefore, no control figures are presented. The patient was given intravenously 4 cc. (0.05 mgm.) of polysaccharide, followed by the administration of 3 cc. (0.03 mgm.) by continuous intravenous drip; 6 days later, the tumor had become sufficiently shrunken and defined to enable the removal of what appeared to be the entire growth. Photographs of 2 different regions are presented in Fig. 61 and 62. Again there is noticeable filamentous extrusion of the chromatin substance from the nuclei, which are almost totally degenerative in the affected areas.

POLYSACCHARIDE COMBINED WITH X-RAYS

Because of a clinical finding that the administration of polysaccharide apparently assisted in breaking down in a few cases the resistance of human tumors to irradiation, we made a few exploratory experiments concerning the effect on mouse sarcomas of combining polysaccharide injection with x-radiation. Mice with 7 day tumors were treated with 0.01 mgm. of polysaccharide, and 4½ hours later the tumors received a single irradiation of 2,500 r. Dual controls were maintained: (a) Animals with tumors treated with
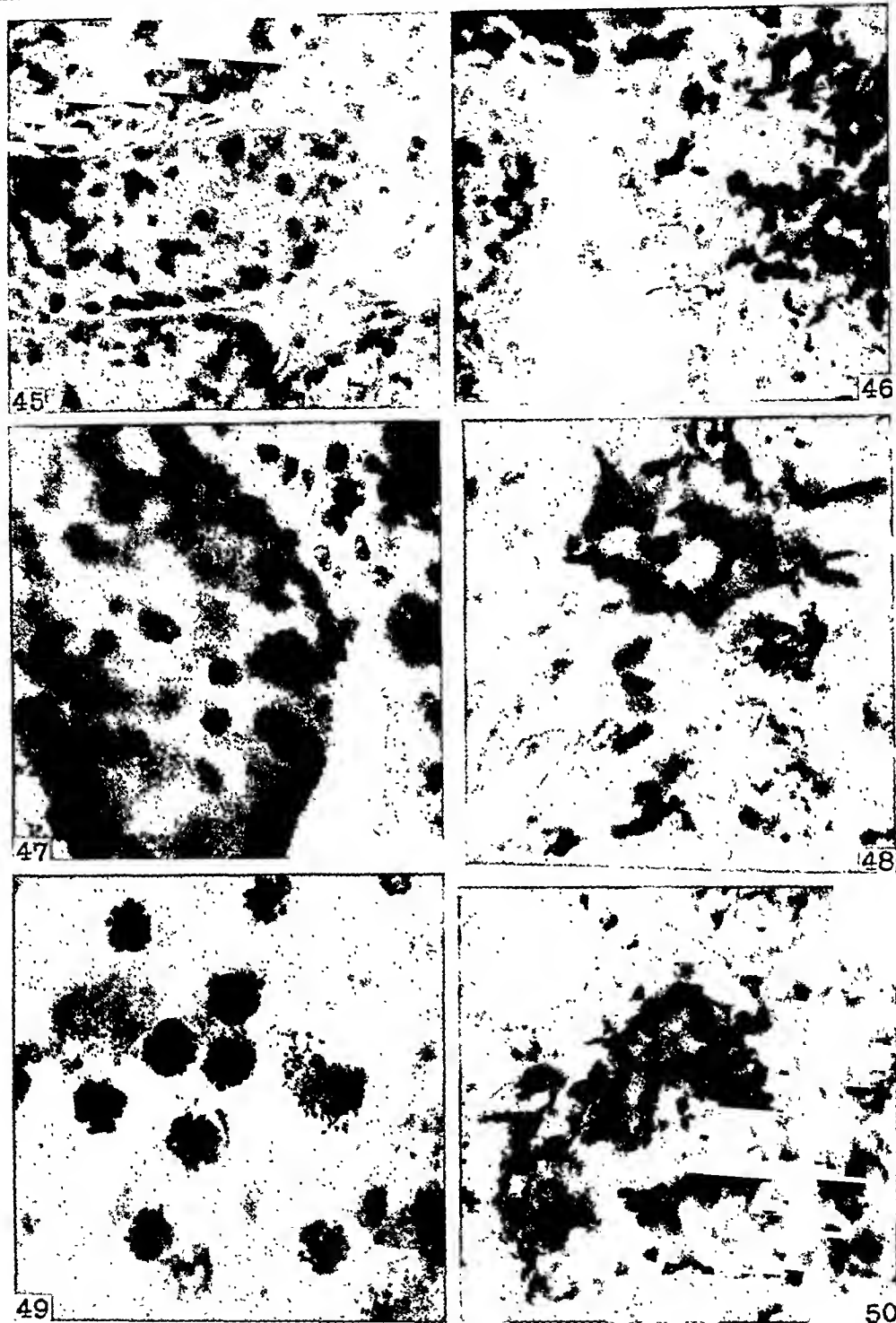
Photomicrographs of acetic orcein smears from normal body tissues of mice without tumors, treated with 0.1 mgm. of *S. marcescens* polysaccharide.

FIGS. 45 and 47.—Low and high power studies of intestinal epithelium of mouse, untreated.

FIGS. 46, 48, and 50.—Low and high power studies of intestinal epithelium of mouse, 6 hours after treatment.

FIG. 49.—Germ cells from mouse testis, undamaged, following administration of 0.1 mgm. of polysaccharide.
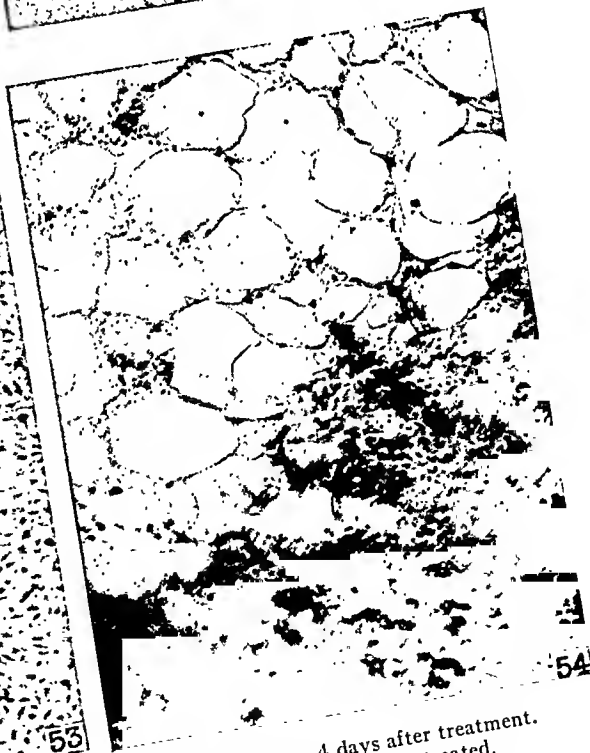
Photomicrographs from paraffin preparations of tumors
in human patients, treated and untreated. Mag × 360.
Fig. 51.—Human chondrosarcoma, untreated.

Fig. 52.—Same, 4 days after treatment.
Fig. 53.—Fibrosarcoma, untreated.
Fig. 54.—Same, 4 days after treatment.

0.01 mgm. of polysaccharide; (b) animals with x-ray-treated tumors only (2,500 r in a single application). All x-rayed mice were placed beneath specially prepared lead shields, in which small openings were cut to allow the tumor to project beyond the shield, and the tumors were treated without removing the hair. The amount of x-ray administered was calculated[2] to be approximately equivalent to 500 r over bare skin.

Some animals were killed for microscopic examination of the tumors, but the remainder were maintained until the tumors were shed, or until their continued growth produced the death of the host. Grossly, about 25 per cent of the tumors responded to polysaccharide alone, as was expected. An almost identical number of degenerates was obtained in the group receiving only x-rays. The combined treatment, however, yielded as high as 75 per cent response, as indicated by total sloughing of the tumors, which showed that the effect was not merely an addition but an enhancement of their combined effects. Microscopic examination revealed that in animals killed at 24 and 72 hours after combined treatment, cellular response, too, was something more than the addition of the two types of damage.

It is well known that cells are most sensitive to x-rays at some stage of mitosis, probably metaphase. Ludford (11) reported that in tumors the mitotic effects of irradiation are greatest on those that are fastest growing. In sarcoma 37 no great change was produced microscopically at 6 hours when x-rays alone were employed; but at 24 hours there was a large amount of degenerating tissue, which apparently arose from chromatin fragmentation. Nevertheless, in only 25 per cent of the animals so treated were the tumors completely destroyed. When the two agents acted together only the expected polysaccharide effects were evident microscopically at 6 hours. However, at 24 hours a combined reaction (polysaccharide degenerates and filaments, plus fragmented chromatin strands) could be detected. The resulting masses of debris were often reduced to fine particles and fragments, as though the filamentous degenerates produced by polysaccharide had also been fragmented by the x-rays. A new phenomenon, moreover, appeared in resting cells. Nuclei of many of these were broken into rather large pieces, which still remained sufficiently in juxtaposition to render it possible to identify them as interkinetic nuclei. Such nuclei are shown in Figs. 21 and 22. The oval contours are lost, the nucleus

is diminished in size, and cross-fragmentation of the entire nucleus has taken place. These changes did not appear in either of the control series. Since both agents act destructively on cells undergoing mitosis, we should not have been surprised had a combined single treatment produced no greater effect than either alone. The observations reported here are of a preliminary nature only, and much more experimentation must be done in order to determine how x-rays and polysaccharide in combination act to overcome the comparative resistance of the resting cell and also in what way minimum amounts of these two drastic agents can be combined for optimum effectiveness in destruction of tumor tissue.

DISCUSSION

The assumption that tumor cells respond directly to the toxic effect of *S. marcescens* polysaccharide and that the resultant degeneration may be independent of or supplementary to the anoxia following breakdown of blood supply appears to be substantiated by the evidence presented. In the first place, it does not seem necessary that hemorrhage occur in order to obtain cellular degeneration, since at the time the initial swelling of the cell begins in response to polysaccharide there may be as yet no trace of hemorrhage within the tumor. Although sarcomas that are completely filled with blood at 6 hours posttreatment usually show a greater amount of necrotic tissue than do those that are only moderately hemorrhagic, nevertheless when nonhemorrhagic areas of tumors are selected for study, the cells are found to be reacting in a fashion identical with that observed in cells from blood-filled areas, except that the coagulation phenomenon is not present.

Care was taken to secure bits of tissue from both hemorrhagic and clear areas in order that microscopic comparisons could be made. In biopsy specimens of large human tumors, the areas of degenerating cells were usually located somewhere in the region of the hemorrhage. However, the presence of blood was not the exclusive criterion of cellular destruction, as was shown when a patient with Hodgkin's disease with multiple cervical lymph node enlargements was treated. Some of these nodes responded by severe hemorrhage, while others did not. Even so, both hemorrhagic and nonhemorrhagic nodes surgically removed after polysaccharide treatment showed cellular response, although it was most extensive in the hemorrhagic nodes.

Furthermore, following polysaccharide administration cell destruction in normal body tissues of

the mouse is not heralded by any marked hemorrhage of tissues; and in intestinal epithelium where damage was greatest, we could never detect macroscopic bloody areas marking the responsive regions, although many blood cells could be found among the degenerating tissues. Neither were there any hemorrhagic patches on liver or spleen following administration of an amount of polysaccharide 10 times as great as that required for tumor necrotization, although destruction of dividing cells in these organs was microscopically demonstrable.

Algire's studies (1) of the vascularization of sarcoma 37 and other tumors led him to observe that "an outstanding characteristic of the tumor cell is its capacity to elicit continually the growth of new capillary endothelium from the host," and Ludford (12) asserted that the vascular damage produced by colchicine in rapidly growing tumors is due to the fact that "the endothelial cells of newly formed capillaries are particularly sensitive to mitotic poisons." A study is now under way to determine, if possible, whether mouse tumor capillaries likewise break down because of polysaccharide destruction of rapidly proliferating endothelial cells. Frequently in rapidly growing tumors, cell multiplication proceeds with a speed with which capillary growth does not keep pace, and the blood travels not in formed capillaries but in spaces surrounded by closely aligned tumor cells. If these tumor cells break down under polysaccharide, hemorrhage naturally follows.

Bearing on these points is the example already mentioned of the stability of capillaries in a less rapidly dividing neoplasm (spontaneous rat carcinoma). In this instance it appeared clear that cellular degeneration was not due to the anoxia incident to deprivation of blood supply.

It can hardly be possible, however, that tumor destruction following polysaccharide treatment is entirely dependent on the direct toxicity to tumor cells; for it is obvious that where lack of oxygen and food supply have occurred through breakdown of the vascular system, there must be additional degeneration of the cells arising from these causes. Moreover, Algire (2) demonstrated by means of the mouse chamber that stasis is observed 3 to 4 hours after polysaccharide, and this may be an added factor in cell degeneration in the earlier stages of response. There is also the possibility that damage to cell walls may result in the leaking of plasma into the intercellular spaces several hours before the red cells come through.

Furthermore, even when some of the tumor cells remain relatively healthy, coagulation of blood and formation of a scab may dispose of some of them by purely mechanical means. That is, the hardened mass of tissue breaks away from the skin and sloughs off, carrying with it some still viable cells mingled with the debris.

On the other hand, we have sometimes observed that tumors which were not sufficiently hemorrhagic to form a hardened mass of blood nevertheless were totally resorbed, indicating that the cells must have been destroyed. These were usually small tumors. In no case was the resorption of a large tumor observed, and this is probably correlated with the larger amount of resting and therefore less responsive tissue present in the older, larger, growths.

Although most of our evidence points to a relatively selective action of the polysaccharide as regards dividing cells, there is no way of determining whether some of the interkinetic cells may not also be affected. However, it is certain that after polysaccharide treatment, cells in mitotic division are destroyed, the mitotic process is suppressed, and the only undamaged nuclei are those of nondividing cells. As shown previously in this report, the first manifestation of polysaccharide effect is a swelling of the prophase nucleus, accompanied by blistering and other changes in the nuclear membrane. This is followed either by collapse of the nuclear membrane or extrusion of the nuclear contents. In the latter case, there is a seeming failure of the swollen nuclei to withstand the pressure of internal fluid, and the nuclear membrane gives way at some point, permitting the solid structures to be extruded. This is the most characteristic feature of nuclear response to polysaccharide and is seldom encountered during spontaneous degeneration. Our colleagues at the National Cancer Institute report that they have noted this type of nuclear degeneration occasionally in untreated tumors, though it is much less extensive than in treated ones, and usually appears in tumors found to be regressing under bacterial contamination.

The factor of spontaneous degeneration of these tumors renders difficult the cytological evaluation of response to chemotherapeutic agents generally; nevertheless in our experimental tumors it was possible to distinguish between induced and spontaneous necrosis with reasonable certainty. For one thing, cells degenerating spontaneously do not exhibit nuclear membrane changes so far as we have been able to observe, and though nuclei become shrunken and pycnotic, no differential degeneration of dividing cells is demonstrable. Nuclei of all stages shrink down into pycnotic spheres which are much smaller than polysaccharide-induced remnants and which show no
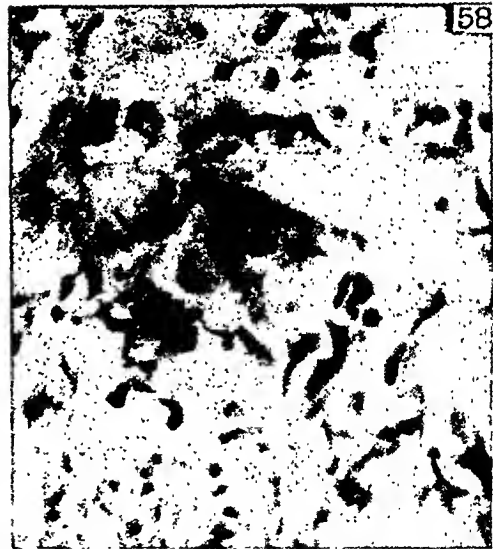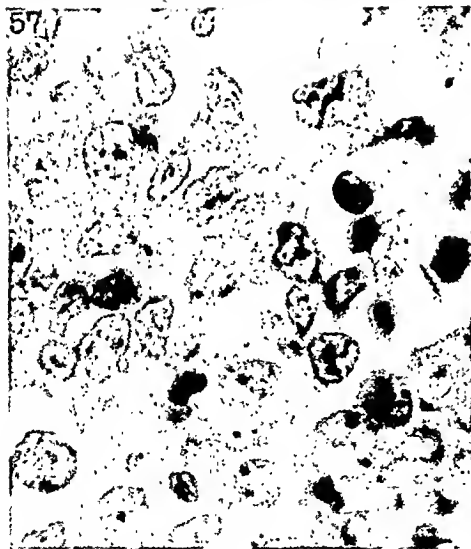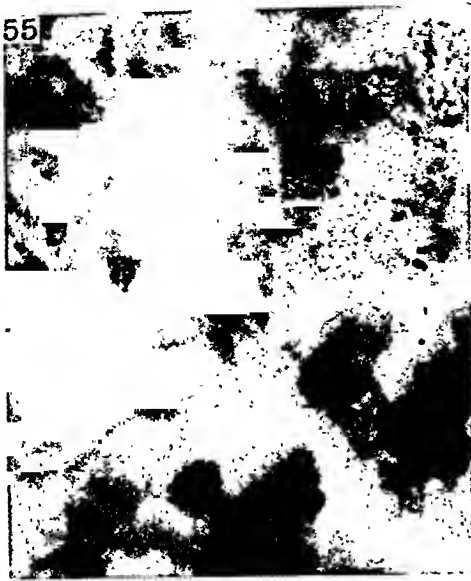
High-power studies (Zeiss 1.5 oil imm. lens) of tumors in human patients, control and treated tissue from biopsies taken before and after treatment. Mag. $\times$ 1,000 (approx.).

FIG. 55.—Human chondrosarcoma, untreated (acetic orcein smear).

FIG. 56.—Same, 4 days after treatment (acetic orcein smear).

FIG. 57.—Human fibrosarcoma, untreated (acetic orcein smear).

FIG. 58.—Same, 4 days after treatment (acetic orcein smear).
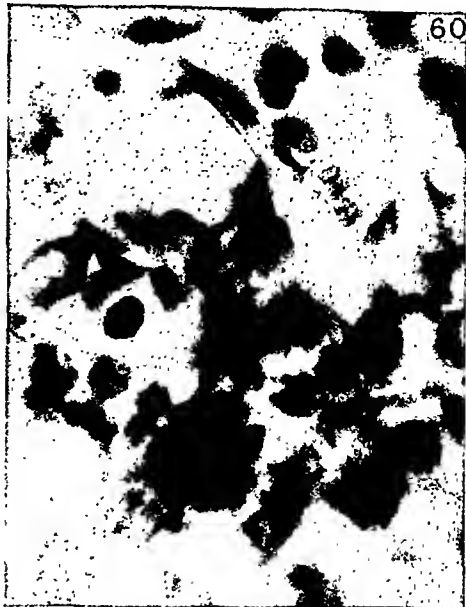
Tumors in human patients, continued.

FIG. 59.—Untreated Hodgkin's lesion from cervical lymph node.

FIG. 60.—Same, 4 days after treatment.

FIGS. 61 and 62.—Human lymphosarcoma, 6 days after treatment, showing different regions of the neoplasm (hemorrhagic and non hemorrhagic).

trace of structure. Areas of spontaneous degeneration are usually filled with droplet-like globules of structureless, strongly orcein-positive substance or minute fragments that are likewise heavily stained and granular in appearance; that is, quite different in size and conformation from the components of cellular debris produced within a few hours after polysaccharide administration.

Following polysaccharide, no blistering or collapse of the nuclear membrane was observed in resting cells, even when they were inhibited from division.

The fact that at each progressive stage of nuclear degeneration following polysaccharide treatment there were present figures characteristic of each of the early stages, suggests that the process of morphologic change follows a rhythm, probably that of division, and that each nucleus runs a gamut of degenerative changes, depending upon the stage of division in which it existed on impact of the polysaccharide.

The assumption that the dividing cell is most responsive is bolstered by the observation that the cells not affected are those in resting stage and also by the reactions of nontumor tissue. Furthermore, the fact that there are no longer any anaphase or telophase figures present when the first of the affected nuclei reach the ultimate in degeneration indicates that mitosis was halted earlier. It should be borne in mind that in neoplasms with such a high mitotic index a large proportion of the cells are in some stage of mitosis at all times.

The reason carcinomas fail to respond as rapidly and vigorously to polysaccharide as do sarcomas is not clear and may of course reside in fundamental chemical differences between the cells of these two types of neoplasm. However, if our supposition is correct that dividing cells are more susceptible than resting cells, there may be a correlation between the restricted response of carcinomas and the small number of dividing cells present in any area of such tumors, as revealed by our controls.

It is uncertain whether cessation of mitosis during the 3 day period following treatment is due to polysaccharide suppression, or whether failure of cells to divide may result from loss of capillary connections. Algire's observations that capillaries are not re-established until the third day following polysaccharide damage may favor the second alternative. Our studies of young implants also bear on this point, since it was observed that wherever there are dividing cells they respond to polysaccharide, and that onset of division is dependent upon establishment of vascular connections.

Moreover, the same phenomena occur as were observed during the first 3 days following implantation and before capillary branching is elicited. That is, there are produced many enlarged nuclei with multiple nucleoli. This suggests that many of the cells continue to carry on the process of duplication of the chromatin through "endomitotic" activity (internal mitosis without nuclear or cell division) postulated in neoplasms by Biesele, Poyner and Painter (5). According to Biesele and his collaborators, such nuclei might theoretically arise in cells with an unusually high concentration of thymonucleoprotein "which might be explained on the basis . . . of synthesis over a long period of time." Such an opportunity for synthesis without chromatin repartition conceivably is afforded during the enforced interkinesis imposed either by polysaccharide inhibition or attendant anoxia.

Our studies of the growth pattern of sarcoma 37 have demonstrated that mitotic activity is not always localized at the periphery but that division centers are scattered throughout the tumor, wherever there is contact with vascular branches. The resultant random distribution of "resting" areas probably accounts for the fact that undamaged fragments of clear tissue, obviously capable of renewed growth, are often macroscopically observable at the periphery of the treated tumor, although the remainder of the growth may have become nothing more than a softened necrotic mass. In collaboration with other members of the Institute, studies are being made on the problem of destroying this persisting tissue through protection of animals against polysaccharide toxicity, breakdown of resistance to repeated treatments, and combination of polysaccharide with x-ray.

SUMMARY

S. marcescens polysaccharide produces nuclear damage to transplanted mouse sarcoma cells, separate from that arising through breakdown of the capillary system. Maximum destruction is attained at 6 hours, and only resting cells persist.

These cells are inhibited from division for 3 days following treatment. During this period they may persist morphologically unaltered, they may undergo some degeneration of cytoplasmic components, or they may become enormously swollen cells with huge nuclei and supernumerary nucleoli.

In a significant number of cases, no viable cells could be detected by our methods 3 days after treatment with a dose that killed 20 to 30 per cent of the mice. The same proportion of tumors was sloughed by animals maintained for survival studies. When polysaccharide was combined with x-rays this number was increased threefold.

Injection directly into the tumor did not inhibit growth.

Repeated treatments did not produce. greater amounts of destruction, indicating that the tumor, or its host, had become resistant to the bacterial agent.

In primary neoplasms, including human sarcomas, the effects were similar to, but much less extensive than, those produced in mouse sarcomas.

ACKNOWLEDGMENTS

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Oral Carcinoma in a Monkey Colony

A Report of Two Additional Cases*

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A search of the literature discloses the extreme rarity of oral carcinomas in domesticated and captive wild animals. Only 15 cases of carcinoma of the tongue in domesticated animals (horse, cow, cat, dog) reported from various countries have come to our attention (14). The autopsy records of the 10,298 mammals and birds that died in the Philadelphia Zoological Garden during a period of about 30 years show that only one animal, a male bear, with a carcinoma of the tongue was found (11). As far as subhuman primates are concerned, the incidence of neoplastic diseases, according to Ratcliffe, has been and still is lower in the Philadelphia Zoological Garden than for any comparable group of mammals (12), and until 1947 no carcinomas of the tongue in monkeys were discovered (13). Nor have we seen any references to malignant neoplasms of the tongue in autopsy reports issued from other laboratories and zoological gardens in which monkeys have come to autopsy. The occurrence of 2 additional oral carcinomas in our laboratory colony of monkeys is of particular interest not only because of the previous occurrence of 3 carcinomas of the tongue in 2 other monkeys of the same colony (14), but also because the 2 cases to be reported involve different sexes and different divisions of the suborder *Pithecoidea*. Furthermore, we believe that the information available on these animals and on the environmental conditions under which the disease occurred is somewhat more detailed than is usually the case in studies of monkeys that have died in laboratories or menageries.

CASE REPORTS

These 2 monkeys were members of a colony of catarrhine and platyrrhine monkeys kept by one of us (H. K.) for neurophysiological and behavioral investigations.

Monkey No. 1.—A male rhesus monkey (*Macaca mulatta*), purchased from an animal dealer in New York City, was received in the laboratory on October 7, 1939. Its age at the time of arrival was estimated to be approximately 8 years. It was kept continuously in the laboratory until its death on February 23, 1945. It is worth noting that throughout this period of observation, whenever the animal was found sitting quietly, its lower jaw drooped (Fig. 1). This behavior is not characteristic of normal rhesus monkeys. During January 1945 the monkey started losing weight and confined itself more and more to eating soft food only. Saliva drooled frequently from its mouth and an occasional twitching in the region of the left corner of the mouth was observed. An examination of the mouth under nembutal anesthesia, on February 22, 1945, revealed an ulcerating lesion at the tip of the tongue measuring about 1.8 cm. in diameter and extending transversely about 2.1 cm. at the under surface of the tongue. Biopsy disclosed a squamous cell carcinoma.

On February 23, 1945, the monkey was anesthetized with ether and then sacrificed by an incision into the heart. Eyes, ears, nose and throat were grossly normal. The tongue had an ulcerating lesion on the left tip extending from the papillary margin down to the floor of the mouth. The submaxillary gland as well as the muscular tissues and vessels on the left side seemed to be directly infiltrated by the tumor. A small isolated nodule was noted in the hard palate. The other submaxillary and sublingual glands as well as the main cervical lymph nodes of the upper part of the neck were involved to a greater or lesser degree by metastatic tumor. The thyroid was not identified. The lungs were normal except for anthracotic pigmentation. The mediastinum and its contents and the heart were normal. Lymph nodes were not involved. The thymus was not identified. The abdominal cavity seemed grossly normal, except for a few adhesions between the viscera, and the spleen was rather small and shrunken. The liver, gallbladder, extrabiliary ducts, pancreas, stomach and small bowel seemed normal. The

large bowel was normal except for a cecal diverticulum. There was a moderate amount of retroperitoneal fat. The kidneys, adrenals, bladder, testes and prostate seemed grossly normal. The teeth were in good alignment and showed no visible caries with the exception of the maxillary third molar on the right side. Body weight: 7.7 kgm.

Microscopic examination of the primary lesion showed a squamous cell carcinoma irradiating deeply into the tongue musculature (Fig. 2). There were regions of anaplasia and regions where

and kept under study in the laboratory until its death on March 1, 1944. It was estimated to be between 2 and 2½ years old at the time of its arrival. On November 6, 1943, a small reddish lesion which at first was not encrusted appeared at the right angle of the mouth. About two weeks later the animal was no longer able or willing to shell peanuts, but only in February 1944 was it apparent that the visible lesion had definitely increased in size with a progressive swelling of the right cheek. The animal was often seen rubbing
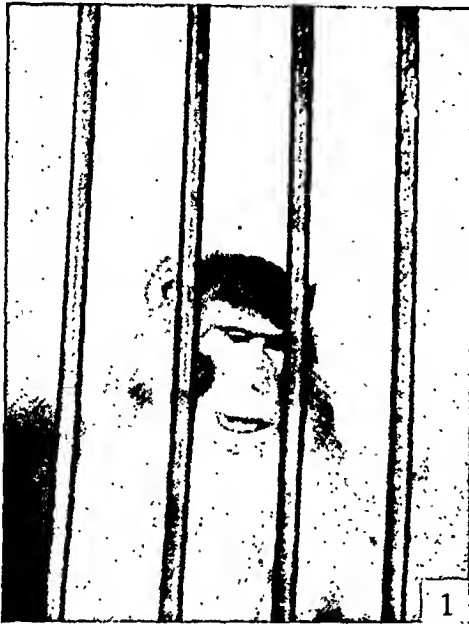
Fig. 1.—Monkey No. 1 (rhesus monkey) showing characteristic drooping of lower jaw.

Fig. 5.—Monkey No. 2 (squirrel monkey) with baby

pearl formation was quite definite. Sections through the enlarged regional cervical nodes showed practically complete replacement of lymphoid tissue by metastatic squamous cell carcinoma (Fig. 3). The following tissues were examined and found to be negative: striated muscle, parotid gland, stomach, small bowel, gallbladder, kidney, adrenal, urinary bladder, pancreas, spleen and lung. The testes showed normal configuration and relationship of tubules and stroma. However, there was definitely less spermatogenesis apparent than would be expected in normal monkeys at the height of sexual potency. The liver showed no cirrhosis or cholangitis. There was extensive fatty infiltration of the liver cells.

Monkey No. 2.—A female squirrel monkey (*Saimiri sciurea*) was received from the Brookfield Zoological Garden (Illinois) on June 3, 1938,

its face against the boards of the cage and scratching its right cheek very gently. On March 1, 1944, the swelling extended from the angle of the mouth to the right ear and was about the size of half a walnut. During the preceding week the animal had not been able to consume any food except milk and milk-soaked bread. When an examination revealed a large ulcerating lesion of the right buccal mucosa emitting an offensive odor, the monkey was killed by means of ether. Body weight: 568 gm. Brain weight: 22 gm. Owing to other demands on the laboratory, no detailed postmortem examination could be made. All organs, including the tongue and the teeth, appeared grossly normal.

Microscopic examination of the lesion of the right buccal mucosa showed a typical squamous cell carcinoma (Fig. 4).

CONDITIONS ASSOCIATED WITH OCCURRENCE
OF CANCERS

Age factor.—At the time of death monkey No. 1 had lived approximately 5 years and 4 months and monkey No. 2, 5 years and 9 months under the conditions of this laboratory. If our estimate of the age on arrival is approximately correct, monkey No. 1 (rhesus) was about 13½ years old at the

death and, within a few days, the death of 3 other squirrel monkeys and of 3 night monkeys housed in the same room. Bacteriological examinations did not reveal the causative organisms. Since this particular female was at least 3 to 4 years old on arrival, we consider it likely that squirrel monkeys may reach an age of 15 to 20 years. It appears, therefore, that monkey No. 2 (squirrel) which was
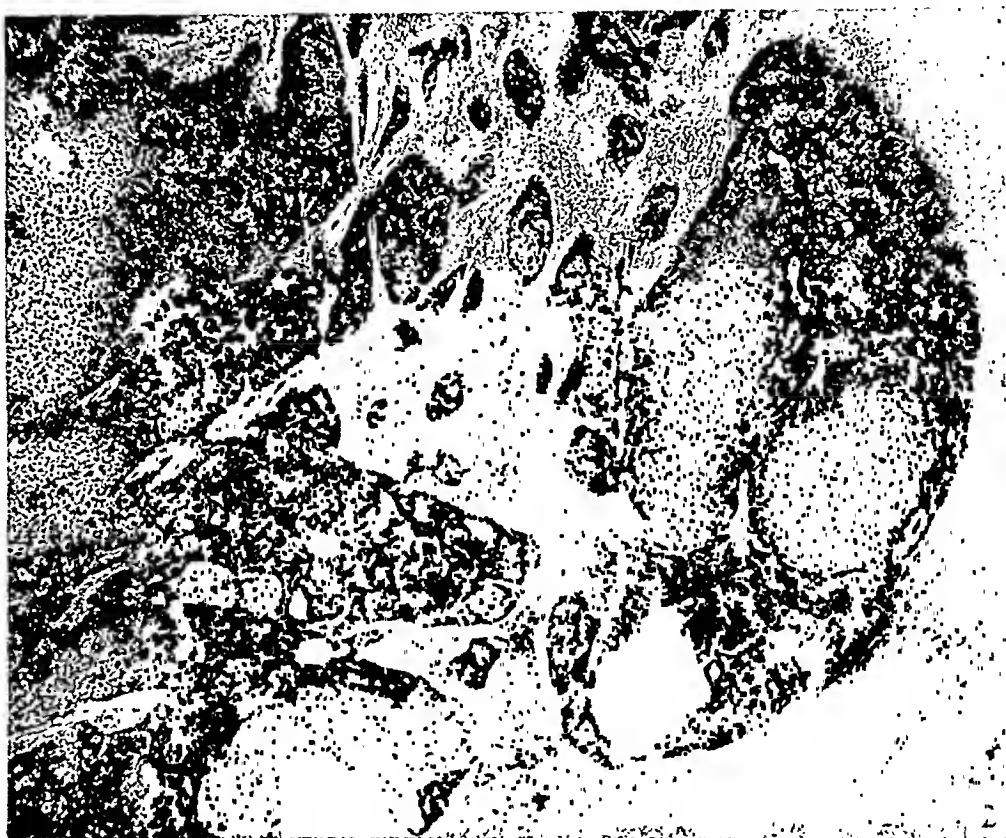
FIG. 2.—Photomicrograph of lesion of the tongue of monkey No. 1 showing typical squamous cell carcinoma with invasion of deeper lymphatics. Mag. × 62.

time of death and must, therefore, be considered aged in view of what is known about the life span of macaques. Published records provide no definite information on the duration of life in squirrel monkeys. Flower (3) discovered that a statement to the effect that a squirrel monkey lived as long as 9 years in the London Zoological Gardens was "founded on a clerical error." However, it is a fact and not a statement based on a clerical error that a female squirrel monkey lived 9 years and 9 months in our laboratory. This animal was apparently in good health and pregnant when an unknown disease suddenly brought about its

only about 8 years old at the time of death cannot be considered aged.

Hereditary factor.—Since monkey No. 1 was an Old World monkey and No. 2 a New World monkey, a common hereditary factor may be safely excluded.

Diet.—The diet used for the monkey colony, except for supplementing it with brewers' yeast, was the same as previously described (14) and appears to have been adequate even for South American monkeys. In view of the fact that in the course of a century only 4 births occurred among South American monkeys in the London Zoolog-

ical Gardens (17), it deserves emphasis that in our laboratory 9 such births occurred during a period of 5 years (1940-1945). Mention should be made of the fact that the daily supply of vegetables and fruits obtained for the animals always included some spoiled and decayed products. Only after cutting off the rotten parts and rinsing or washing the rest under running water was such food offered to the animals. However, there is no doubt

during the first 3 months. In fact, the gain in weight continued so that about 3 years later the monkey was fairly obese and started losing weight only during the last weeks of its life. Nevertheless, it did not always enjoy perfect health since it was at intervals subject to diarrhea and was now and then seen lying on the seat of its cage. Its dull expression and its slow movements when undisturbed made an observer easily forget that it was
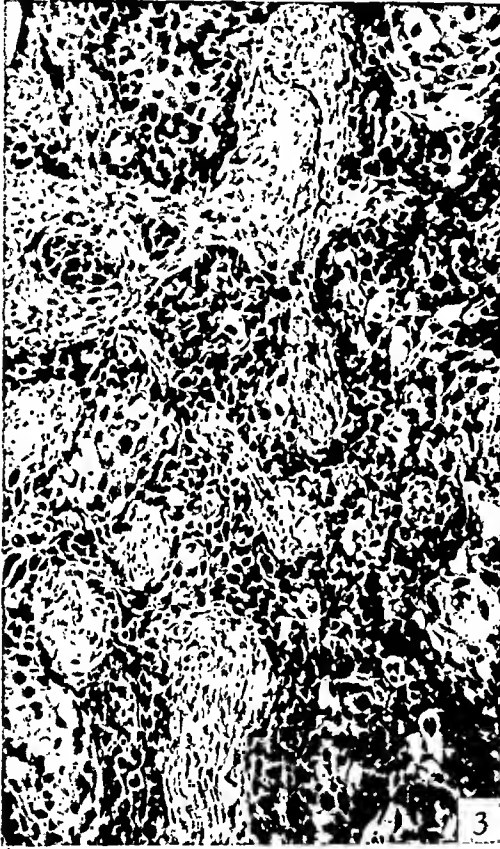
FIG. 3.—Photomicrograph of metastatic squamous cell carcinoma in cervical lymph node from primary lesion shown in Fig. 2. Mag. × 125.

FIG. 4.—Photomicrograph of primary lesion of the buccal muco a of monkey No. 2 showing typical infiltrating squamous cell carcinoma. Mag. × 77.

that the removal of the visible rotten parts was not always complete and that the monkeys, throughout the years of their existence in the laboratory, often chewed and even consumed decomposed matter of plant origin. Although no hot food was ever offered, boiled food (potatoes) was at times part of the diet of the Old World monkeys.

When received in the laboratory, monkey No. 1 appeared to be somewhat emaciated and in rather poor health. It was listless and frequently had diarrhea, but with the care and diet given in the laboratory its general condition rapidly improved

a powerful and aggressive animal ever ready to exhibit startling speed and agility in attacking any human being within reach and even succeeding in seriously injuring an experienced caretaker. As a rule, normal rhesus monkeys do not accept or eat meat offered to them although fully mature or old animals recently arrived from India may occasionally do so. In testing the food preferences of monkey No. 1 it was observed that the animal accepted and slowly ate pieces of sausages or boiled tongue. It seems likely that it had been forced at times to rely on animal products for food in its natural habitat, where it probably also ac-

quired its extreme fear of snakes which extended to toy snakes and wavy lines drawn on cardboard. In connection with dietary habits it is of further interest that the animal when thirsty would drink a 10 per cent NaCl solution, but not a saturated aqueous solution of quinine sulfate (as done by some apparently healthy monkeys).

Monkey No. 2 appeared to be in excellent health from the very beginning of its life in the laboratory. Although squirrel monkeys in captivity readily consume numerous animal products, no meat was included in the diet provided for monkey No. 2. However, it was given a raw egg once a week and it supplemented this diet by eating the live cockroaches it caught every day. In view of the fact that all normal monkeys of the colony thrived under these conditions as indicated by general appearance, behavior, longevity, the occurrence of births, the growth curves and general condition of the offspring, it seems unlikely that the lesion at the right angle of the mouth in monkey No. 2 was the result of nutritional deficiency.

Brain lesions.—Since the previously reported carcinomas occurred in monkeys with brain lesions (14), it is of special interest that one of the two cases reported here had also undergone a brain operation. On January 24, 1941, monkey No. 1 underwent extirpation of the left temporal lobe including most of the amygdala, hippocampus and uncus. Anesthesia was induced by an intraperitoneal injection of nembutal. The Bovie high frequency current and blunt spatulae were used in connection with the operation. There was an uneventful recovery.

Hormonal and sex factors.—Throughout the years of its laboratory existence monkey No. 1 exhibited a red coloration, unaccompanied by swelling, of the "sexual skin," including the scrotal skin. There was only an occasional blanching, but at times there was also a reddening as intense as that seen in pregnant females of this species. Incidentally, the same intensive reddening may at the present time be observed in all aged or old male rhesus monkeys of our colony. In monkey No. 1, no overt sexual behavior was ever observed except masturbation during the week following the brain operation. It is known that the reddening and swelling of the sexual skin in the female rhesus monkey develops progressively during the follicular phase (1, 18, 19) and that the inhibitory effects of progesterone on the effects of estrogen on the sexual skin are particularly marked in juvenile monkeys (1). The response of the region of the sexual skin in normal male rhesus monkeys seems to be similar to that seen in females (16,19). Zuckerman (18) has considered the possibility that

the activating agent of the sexual skin in male monkeys is not an estrogenic substance. It is of interest in this connection that Dr. F. C. Koch found 6 international units of androgens (1.22 units/kgm. of body weight) per 24 hours and no estrogens in capon assays on the urine of a sexually mature male rhesus monkey of our colony. However, there was no reddening of the sexual skin at the time the assays were made and red coloration developed only years later. It will be necessary to study the daily urinary excretion of androgens and estrogens in male rhesus monkeys exhibiting continuously a brilliant sexual skin.

In the case of monkey No. 2, the period of visible development of lesion and tumor fell into a period of gestation and lactation. The first signs of pregnancy became manifest 7 weeks before the appearance of the small lesion at the right angle of the mouth. Monkey No. 2 gave birth to a male baby on November 24, 1943 (Fig. 5). We have not seen any published records indicating that birth of a squirrel monkey has ever previously occurred in captivity. Nursing and maternal behavior as well as growth of the baby seemed to be normal in all respects. The infant was only about 14 weeks old when it became necessary to sacrifice the mother, but its development was such that it continued to thrive on solid food and milk.

Infectious and contagious factors.—The 2 monkeys were housed in different rooms of the same building. The cage in which monkey No. 1 was always kept alone stood in a room used as quarters for rhesus monkeys and a number of monkeys belonging to other species. Monkey No. 2 always occupied a cage together with the other squirrel monkeys of the colony. The same room contained cages housing night monkeys and rats. All animals were exposed to the same caretakers and investigators. There was no evidence of tuberculosis or other diseases in the colony during the years monkeys No. 1 and No. 2 lived in the laboratory.

Porphyrins.—In both monkeys the ulcerated portions of the carcinomas exhibited a striking red fluorescence when they were illuminated by light from a mercury vapor lamp passing through a Corning filter No. 5874. The fluorescence spectra of these tissues suggested the occurrence of free porphyrins. In monkey No. 1 the red fluorescence as well as a fluorescence spectrum characteristic of porphyrins were seen only in the cancer tissue of the tongue, but not in the metastatic tumor. In monkey No. 2 it was the surface of the lesion involving the right buccal mucosa which exhibited the most brilliant red fluorescence. When an examination of a cross section through the right cheek was made, it was apparent that the red

fluorescence became less intense with increasing distance from the ulcerated surface and that it finally changed into a violet and whitish-violet fluorescence. The lesion at the right angle of the mouth, even after removal of its encrustation, showed a deep red fluorescence. In both monkeys, small portions of the tissues exhibiting red fluorescence were removed for spectrochemical investigation. An extraction of the tissues provided evidence, chiefly based on solubilities and the measurements of fluorescence spectra in different solvents, that the cancer tissue from monkey No. 1 contained deuteroporphyrin and that from No. 2, protoporphyrin. In addition, traces of other ether-soluble porphyrins were present, but the amounts were not sufficient for identification (probably protoporphyrin in the case of monkey No. 1 and coproporphyrin in No. 2). The fluorescence of cancer tissues has engaged the attention of several investigators (2, 4, 5, 7-10, 15). Although porphyrins have been held responsible for the red fluorescence occasionally seen in cancers, it seems that such porphyrins have not been isolated and identified by extracting the cancerous tissues. In examining rat sarcomas, Policard (10) observed that the necrotic center of the inoculation tumor showed an intense red fluorescence surrounded by a narrow violet zone which was again encircled by a peripheral zone showing white fluorescence. Körbler (7, 8) described and photographed the red fluorescence of human ulcerated carcinomas. He was not able, and Policard did not even try, to isolate the porphyrin supposedly responsible for the red fluorescence. Both investigators believed hematoporphyrin to be the agent responsible for the effects observed, but we know at the present time that it is not one of the naturally occurring porphyrins. In considering the origin of the porphyrins found in the ulcerated cancer tissues of monkeys No. 1 and 2, it seems safe to consider, first of all, such factors as bacterial action and the degradation of blood and food remnants. We have previously reported that an intense red fluorescence may be observed in the necrotic wounds of monkeys and that various porphyrins may be extracted from necrotic tissue (6). Neither the extractions of portions of the metastatic tumor in monkey No. 1 nor the spectroscopic examination of the non-ulcerated portions of the tumor in monkey No. 2 provided any definite evidence for the occurrence of porphyrins. It is worth mentioning that the tartar found on the molars of monkey No. 1 exhibited a red fluorescence and a fluorescence spectrum with an emission band at about 620 mμ. Since we have made similar observations on many normal monkeys it is doubtful whether any special significance

can be attached to the finding. Extractions of such deposits always furnish one or several ether-soluble porphyrins.

Various environmental factors.—The conditions prevailing in the laboratory as to temperature, illumination, housing, and food utensils have been previously described (14). The animals were under close observation, but no injuries of the tongue were ever noticed. The food never included such items as oats and barley. With exception of the anesthetics mentioned, no drugs were ever administered. Other animals present at various times in the monkey quarters of the laboratory were cockroaches, mice, cats and human beings. Since the laboratory building was once used for work in chemistry, it was considered desirable to check on the presence of radioactivity. Dr. Nickson from the Argonne National Laboratory reported that the spot checks around monkey cages, sinks, door knobs, floors, walls and cabinets failed to reveal any alpha, beta or gamma radioactivity exceeding 100 alpha counts per minute per 100 sq. cm., or 0.1 mr. per hour at 1 inch.

SUMMARY

Two squamous cell carcinomas occurred in 2 monkeys of a laboratory colony in which 3 squamous cell carcinomas of the tongue in 2 other monkeys had previously been observed. A squamous cell carcinoma of the tongue with local tumor metastases occurred in a male rhesus monkey and a squamous cell carcinoma of the right cheek in a female squirrel monkey. The period of life in the laboratory was approximately 5½ years for both monkeys. The South American squirrel monkey was not considered aged at the time of death. The male rhesus monkey, an animal with an experimental brain lesion of long standing, exhibited an almost continuous reddening of the "sexual skin." In the squirrel monkey the period of the visible development of the tumor fell into a period of gestation and lactation. In both monkeys the ulcerated portions of the carcinomas showed a striking red fluorescence. The porphyrins isolated from the ulcerated tissues were deuteroporphyrin and protoporphyrin. The occurrence of the disease could not be definitely related to any of the etiological factors supposedly responsible for oral carcinomas in man. The fact that of the 30 monkeys in the stock colony of the laboratory 4 have been found to have oral carcinoma (a gross incidence of 13 per cent of this population) and that monkeys belonging to species as different as *Macaca mulatta* and *Saimiri sciurea* have been among the affected animals strongly suggests that an extraneous factor may have played an etio-

logical role although there is no indication as yet what this factor may be.

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Antibody Response to Tumor Inoculation in Mice

With Special Reference to Partial Antibodies*

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INTRODUCTION

The present writer (8–10) has advanced the theory that the genes determining the fate of transplanted tissues are identical with those determining the presence of iso-antigenic differences. Such differences are most easily studied in erythrocytes. Strong's A strain happens to possess two iso-antigens that are shared by the erythrocytes and a number of fixed tissues, including all neoplastic tissues studied so far. These antigens have been called antigens I and II, the latter being the more potent. The best antibody producers found to date have been members of Little's C57 black strain.

A spindle cell sarcoma and a myeloblastic leukemia arising in the A strain have been tested on hybrids between these two strains. In each case it appeared that 2 dominant genes were essential for growth, one of which appeared to be identical with the gene for antigen II. Sera for the detection of antigen I are difficult to obtain and were not available for the genetical investigations. Mice in which the tumors had regressed produced iso-agglutinins; in most cases these could be identified as "anti-II," but certain sera contained an additional agglutinin that reacted with the cells of mice of the CBA strain, which shares antigen I with the A strain but lacks II. It seems likely, therefore that both neoplasms contain antigens I and II (9). Agglutinins may also be produced by inoculation of blood or normal tissues, neoplastic tissues giving the better response, partly perhaps because of their greater proliferative power, but possibly because they contain more of the pertinent antigens than normal tissues (9, 10).

The results summarized in the preceeding paragraph were obtained with animals that had been inoculated once. The response to hyper-immunization was studied principally in connection with leukemia. Here it appeared that following two or more injections, the hemagglutinins disappeared. However, sera that were apparently free from agglutinins could protect mice of the A strain against inoculation with leukemic cells, as could those from which the agglutinins had been removed by absorption. It was deduced that mice produced two sorts of antibody, agglutinins and protective antibodies. The latter could be partially absorbed by red cells but far less readily than by leukemic cells, indeed the absorption by the former was difficult to detect. However, the agglutinins are far more easily absorbed by neoplastic cells than by erythrocytes and the conclusion was reached that whilst the two antibodies were qualitatively distinct, they were both directed against the same antigens (10).

At this point the investigations were interrupted by the war. In the meantime, important advances have been made in the study of iso-immunization to the rhesus antigens in man. Here again it appears that two qualitatively distinct antibodies may be formed, ordinary agglutinins and partial or blocking antibodies (11, 13). The latter need special methods for detection *in vitro*, reference to which will be made below, but they appear to be of greater functional significance and are sometimes referred to as the mature type of Rh antibody (3, 15). This situation shows interesting analogies to that just described for mice and we, therefore, decided to see whether such antibodies could be detected in this species.

MATERIALS AND METHODS

The Roscoe B. Jackson Memorial Laboratory had two A strain tumors available, known as 15091a and C1300. The former has been described as a spindle cell mammary carcinoma. It is a pleomorphic anaplastic growth with fairly numerous giant cells. The latter is a round cell tumor, possibly a neuroblastoma. Of the two, 15091a is much more virulent for heterologous pure strains, causing a high mortality in many of them. In the

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C57 black mice it seldom causes death but far larger masses are usually produced than is the case with C1300. They were inoculated in the ordinary way by trocar and canula.

Previous experience has shown that the agglutination tests must be performed in tubes and that in reading, care must be taken to avoid too much force in breaking up the cellular deposit. The author (7, 9) has found the following method suitable: About 0.05 cc. of serum dilution and 1 per cent cell suspensions are set up in dwarf test tubes and incubated for an hour or more at 37°C. The tubes are then centrifuged for 2 minutes at about 700 r.p.m. (it is important that spinning should not be too violent or too long). The readings are then made by *gently* pumping the supernatant back and forth with a capillary pipette. In the absence of agglutination the cells come away as an even cloud, sometimes leaving a small wisp of cells attached to the tube. After pumping 4 times most of the deposit should be resuspended If there is feeble agglutination, the deposit may be easily resuspended but has a granular appearance. As the strength of the reaction increases, the deposit breaks up into lumps of increasing size and often comes off as a solid pellet. There is also a tendency for agglutinated cells to stick to the glass, and this in itself is often a helpful criterion. There is only one real source of confusion in microscopical examination. In controls one may often see a few large clumps of cells. If the slide is gently tilted these will break up, the cells coming away singly. If they come away in small clumps there is some agglutination.

When doing tests where the final concentration of serum or plasma is relatively low, it is not necessary to wash the cells. If the final concentration of serum or plasma is high (say 50 per cent or over) washing is essential. It has been found most satisfactory to perform the washing with the 1 per cent suspensions. These are centrifuged at about 700 r.p.m. Two such washings are sufficient although 3 have sometimes been used. If the cells are not washed but suspended in pure serum, the whole mixture may clot, or partial clotting may occur, closely minicking agglutination.

For the detection of partial antibodies in man the following technics have been used: the blocking test (11, 13), the conglutinin test of Wiener (14), the anti-globulin test of Coombs, Mourant and Race (2), the open slide (4) and albumin tests of Diamond and his co-workers (6). All of these have been tested. The last two seem inapplicable to mouse antisera. Human albumin was used and found to be lytic for mouse cells in saline; the lysis was inhibited by serum, but it did not bring about agglutination by blocking antibodies. The Coombs test gave some suggestive results and will have to be retested with more potent sera.

The technic of the blocking test will be described below. Wiener's conglutinin test depends upon the performance of all operations in compatible serum or plasma. This has been used successfully although the results are slightly different from those obtained with human sera. In this case it has not been found essential to avoid the slightest dilution of serum. When testing some sera the cells have been suspended in saline and all antibody dilutions done in compatible serum. This gives a final concentration of serum of 50 per cent. The most satisfactory system for general use has been with the serum dilutions as before but with the cells suspended in 50 per cent serum, giving a final concentration of 75 per cent serum. Where the standard method has been used, it is referred to simply as "saline." Except where stated to the contrary, pooled sera from 6 mice were titrated.

RESULTS

The Detection of Antibodies in Normal Serum

Up to the present it has been generally agreed that mice do not possess natural iso-agglutinins. It was, therefore, surprising to find that sera of C57 blacks would often cause agglutination of A strain erythrocytes.[1] At first it was thought that this might be non-specific agglutination due to some undetected technical error. This is not the case since C57 black cells are not agglutinated. Originally the discovery was made with pooled sera from 6 or more mice. Later it was found that about 20 per cent of blacks gave some agglutination. Usually the reactions were feeble and could only be detected if the cells were suspended in the sera. However, in one pool strong agglutination was found. The antibody reacted with the cells of strains A, C3H and dba, but not with those of Bagg albino C, or the C57 blacks.

At the present time it is not possible to say what stimulus elicits these iso-antibodies. They have been found in both sexes, but it is possible that fetuses containing the pertinent antigens may sometimes stimulate their formation. This aspect of the problem would probably repay investigation.

The natural iso-antibodies can easily be absorbed, but it has been found more convenient to use the sera of strain A or C3H animals as a vehicle, and this has been done in the experiments to be reported below.

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[1] This has been found to be true of Swiss mice also.

THE ANTIBODY RESPONSE TO BLOOD

It is well known that no two tumors are exactly alike and that a given tumor may undergo variation. Of the normal tissues, blood has many advantages and a few preliminary experiments were therefore performed in order to see if the results were of the same kind as those obtained previously. Citrated whole blood was inoculated intraperitoneally, allowing for the dilution about 0.25 cc. were given at each inoculation. In the first experiment (see Table I) the inoculations were given at weekly intervals. The mice were bled after the first, second and fourth inoculations. In the second

the subsequent ones whenever the tumor is ready to transfer. Probably about 10 day intervals are the best, but one can get almost the same result if the animals are rested a month or more and then given a booster inoculation.

The first four sera shown in Table II were all from the same group of animals. This is the type of response that may be taken as typical. Here again we see an apparent disappearance of agglutinins following a second inoculation. However, in this case titration in serum shows some agglutination with complete inhibition in the prozone. Following further inoculations there is a

TABLE I: THE ANTIBODY RESPONSE TO WHOLE BLOOD

Dose of blood given, cc.	No. of days since last inoculation	Technic*	Dilution of serum									Remarks
			2	4	8	16	32	64	128	256	512	
0.25	7	Saline	+++	++	±	tr	—	—	—	—	—	Dosage at weekly intervals
		75% serum	a.c.	+++	+++	++	—	—	—	—	—	
0.5	7	Saline	+	—	—	—	—	—	—	—	—	
		75% serum	+±	+±	—	—	—	—	—	—	—	
1.0	7	Saline	+++	+++	c	c	c	a.c.	a.c.	+	—	
		75% serum	+++	a.c.	c	a.c.	a.c.	a.c.	a.c.	tr	—	
1.0	10	Saline	c	+++	++	tr	—	—	—	—	—	Dosage twice weekly
		75% serum	c	a.c.	+++	+	—	—	—	—	—	
2.0	10	Saline	++	a.c.	c	+++	++	+	tr	—	—	
		75% serum	++	a.c.	a.c.	a.c.	+++	+++	++	+	±	

*The meaning of the terms in this column are explained in the text.
c = complete agglutination; a.c., almost complete agglutination; tr, trace, etc.

experiment the mice were injected twice a week until about 1.0 cc. had been given, when they were bled. They were rested for three weeks and then given two inoculations of 0.5 cc. within a week. The objective of the second experiment was to keep the mice constantly flooded with antigen as probably occurs with a tumor that proliferates and then regresses.

The first series is perhaps the most instructive. It will be seen that following the second 0.25 cc. there is an apparent drop in the titer of antibodies, to be followed by a rise in titer after 1.0 cc. had been given. Comparison with the second series shows that the response is less good when 1.0 cc. is given in concentrated dosage over 2 weeks than when the course is spread over a month.

Normal serum did not enhance agglutination to any great extent in these experiments. It will be noticed that there is a suggestion of a pro-zone with the hyper-immune sera.

THE RESPONSE TO TUMOR C1300

As is the case with blood, the spacing of the inoculations has an influence on the result. A short, intensive course of inoculations gave a lower titer than when they were spread out (see last titer, Table II). As a rule it is best to allow about 2 weeks to elapse following the first inoculation and give

reappearance of agglutination in saline but the inhibition zone remains. This is almost invariably 1 tube shorter in serum. Further inoculations raise the titer and the inhibition zone tends to become shorter. In one group of animals it had disappeared after 6 inoculations. This is by no means always the case, however. An inhibition zone up to about ⅛ usually remains. Increasing the number of inoculations still further may sometimes cause an apparent drop in titer.

The fifth titration shows the effects of prolonging the interval between inoculation and bleeding. In that illustrated in Table II all agglutinins active in saline have disappeared, whilst in serum there is a fair titer with a marked inhibition zone. In another case there were apparently no antibodies at 21 days. However, this particular serum gave a strongly positive blocking test.

These experiments indicate that there are 3 types of antibody produced: ordinary agglutinins, agglutinins needing normal serum for their activity, and blocking antibodies.

THE RESPONSE TO TUMOR 15091a

This tumor has been studied in C57 brown as well as black mice. The latter are the more resistant but even here the response to primary inoculation is variable. The animals were usually inoculated

in groups of 12. Sometimes 2 or 3 of these died from the tumor. Thereafter 2 or 3 more groups were inoculated without a single death and with complete regression in about 2 weeks. This has been the common experience in recent months. The response to primary inoculation has been studied in the C57 blacks only. If there are persistent growths, one gets a serum with a marked inhibition zone as shown in the first entry serum in Table III. Following hyperimmunization in C57 blacks one does not get an apparent disappearance of antibodies, as occurred with the other two antigens. If the inoculations are given at very short

mentioned. Similar antibodies have been observed in the serum of animals that had recieved a slow-growing mammary carcinoma from the C3H strain. A more interesting example is given by animals that had been inoculated with doses of 5 mgm. of lyophilized 15091a. They showed a titer of 16 in serum but no agglutination in saline. As has been mentioned previously, Snell has shown that this treatment renders the animals highly susceptible to the living growth. Whilst the antibody response is feeble, it is interesting to note that it has not been completely inhibited.

Plasma may be used instead of serum, but it is

TABLE II: THE RESPONSE OF C57 BLACKS TO TUMOR C1300

No of injections	Day of bleeding	Titred in	2	4	8	16	32	64	128	256	512	1024	Notes
1	14	Saline	c	a.c.	+++	tr	~	−	−	−	~	−	First four titers, same group
		75% serum	c	c	+++	+++	tr	~	−	−	−	−	of animals
2	10	Saline	−	−	−	−	~	−	−	−	−	−	
		75% serum	−	−	−	−	tr	±	±	tr	tr	tr	
4	10	Saline	~	−	−	++	++	±	tr	tr	−		
		75% serum	~	−	−	++	+++	+++	+++	a.c.	+++	±	
4	23	Saline	~	−	−	−	c	−	−	−	−	−	
		75% serum	−	−	++	a.c.	c	+++	++	+	tr	−	
4	10	Saline	+++	a.c.	+++	++	~	−	−	−	−	−	Eighteen days between 1st
		75% serum	c	a.c	+++	++	+	−	−	−	−	~	and 2nd inoculation. Last 3 at 3 day intervals.

intervals, one may get an inhibition zone similar to that occurring with C1300 (fifth serum, Table III). If they are spaced out further one seldom gets a pro-zone at all. If there is one, there is only partial inhibition of agglutination.

One black mouse which was apparently dying of a large fungating growth, had blocking antibodies only. Dr. Snell was kind enough to give me some mice that had been rendered artificially susceptible by being inoculated with a lyophilized preparation of 15091a prior to the living tumor (12). These also were obviously *in extremis*. Their pooled sera gave the highest titer seen to date. It was over 16,000 in saline but only 4,096 in serum. This is an unusual finding.

It will be noticed that the ordinary agglutinins persist longer following inoculation with this growth than they do with C1300 (fourth serum, Table III).

Hyperimmunized C57 brown mice always give a pro-zone with this tumor. This was especially pronounced with serum of 2 animals with persistent growths. Two similar mice were tested individually. One had a smaller pro-zone than those shown in the table, with a titer of more than 4,096. The other showed blocking antibodies only.

ANTIBODIES NEEDING ENHANCEMENT
WITH NORMAL SERUM

As can be seen from Table I to III, in most cases agglutination is inhanced by normal serum. Two cases in which it is essential have already been

awkward to store and cannot be used for experiments on the effects of heat. The enhancing factor (or factors) is thermostable, appearing to be undamaged by exposure to 60°C. for 30 minutes. As a rule the serum is used almost as soon as it is available. One sample that had been stored in the frozen state for a month did not appear quite so good as a fresh sample. It is perfectly safe to lay in a week's supply at a time.

The amount of serum needed appears to depend upon the concentration of antibody. With natural antibodies one may get agglutination with 70 per cent. With feeble antigens such as the C3H carcinoma, the titer will be appreciably lower in 50 per cent serum than in 75 per cent serum. With some of the anti-C1300 sera, one may get some agglutination with as little as 10 per cent. It is obvious that the situation is different from that found by Wiener with human serum where it is essential to avoid any dilution. Furthermore, mouse serum will not cause agglutination with true blocking antibodies.

THE DETECTION OF BLOCKING ANTIBODIES

Blocking tests were first performed by Wiener and Race (11, 13) in connection with Rh sensitization, although Coca and Kelley had described essentially the same phenomenon in 1921 (1).

There are various possible modifications in the technic. In the present series of experiments two methods of titrating the sera were tried. In the first the test sera were diluted as in an ordinary

titration, incubated with red cells and a given quantity of agglutinin added to each tube. In the second case, undiluted immune sera were incubated together with red cells and varying quantities of agglutinin added. Examples of both of these are shown in Tables IV and V. Neither are ideal for titrating sera, although the latter appears preferable.

The first method has certain theoretical interest. The occurrence of a pro-zone suggests certain analogies with the inhibition due to antibody excess such as occurs with certain precipitating antisera from horses. It is difficult to check this with

TABLE V: BLOCKING TEST

Blocking serum undiluted. Agglutinin varied.

Cells incubated in:	Dilution of Agglutinin Added				
	2	4	8	16	32
Saline	++	++	+	+	—
Normal A serum	+++	+++	++	++	+
Blocking serum	—	—	—	—	—

The blocking serum used here was anti-C15091a from an animal with a tumor. There was not enough serum for further tests.

this is done the pro-zone is invariably reduced, the reduction being greater with increasing amounts of added antibody, as is clearly shown in Table IV. This is the opposite of what would occur if the inhibition were due to antibody excess.

TABLE III: THE RESPONSE TO TUMOR 15091a
(A) C57 BLACK

No. of inocu-lations	Day of bleed-ing	Titred in	Dilution of serum											Notes
			2	4	8	16	32	64	128	256	512	1024	2048	4096	
1	51	Saline	—	—	—	—	++	+++	—	—	—	—	—	—	Delayed regression. 1 mouse subsequently died.
		75% serum	—	—	—	+	++	+++	++	+	—	—	—	—	
1	15	Saline	c	c	a.c.	+++	+	±	tr	—	—	—	—	—	
		75% serum	c	c	+++	+++	++	+	tr	—	—	—	—	—	
3	11	Saline	c	c	c	c	c	c	+++	+++	++	—	—	—	
		75% serum	±	c	c	c	c	c	a.c.	+++	++	—	—	—	
4	52	Saline	+++	++	++	++	++	±	—	—	—	—	—	—	
		75% serum	a.c.	a.c.	a.c.	+++	+++	+++	+++	++	—	—	—	—	
4	10	Saline	—	—	—	—	—	+++	+++	++±	++±	—	—	—	15 days between 1st and 2nd inoculation. Others at 3 day intervals
		75% serum	—	—	—	—	—	+++	a.c.	a.c.	c	+++	+	—	
1		Saline	c	c	c	c	c	c	c	c	c	c	c	c	Animals with huge growths. See text
		75% serum	c	c	c	c	c	c	c	c	c	+++	++	+	

(B) C57 BROWN

No. of inocu-lations	Day of bleed-ing	Titred in	2	4	8	16	32	64	128	256	512	1024	2048	4096	Notes
3	12	Saline	—	—	—	—	—	—	+	±	±	tr	—	—	Tumors regressed.
		75% serum	—	—	—	—	—	++	+++	a.c.	c	a.c.	Not tested		
3	12	Saline	—	—	—	—	—	—	—	—	++	+++	Not tested		Large tumors. Serum from 2 mice pooled.
		75% serum	—	—	—	—	—	—	—	+	++	a.c.	Not tested		
4	21	75% serum	—	—	—	—	—	—	+	+++	+++	+++	+++	++±	Large tumor. Individual tested.
3	33	Saline	—	—	—	—	—	—	—	—	—	—	—	—	Large tumor. Positive blocking test. 1 individual tested. See Table V.
		75% serum	—	—	—	—	—	—	—	—	—	—	—	—	

excess antigen, since with very heavy cell suspensions the results are difficult to read. With suspensions up to 10 per cent the pro-zone did not appear to be shortened nor could this be done by adding light suspensions of malignant cells. However, in both these cases it is possible that insufficient antigen was added. Therefore, it seemed easier to approach the matter from another angle and see the effect of adding excess antibody. If

TABLE IV: BLOCKING TEST

Blocking serum diluted.
Constant amount of agglutinin added.

Agglutinin added	Dilution of Blocking Serum								
	1	2	4	8	16	32	64	128	256
Nil	—	—	—	—	—	—	±	±	±
Anti-C1300.									
4 units	—	—	—	±	+	c	c	Not read	
Anti-15091a.									
2 units	—	—	—	?	±	+++	+++	"	"

Controls: Saline + anti-C1300 (4 units) +++
Saline + anti-15091a (2 units) +

THE EFFECT OF STORAGE ON SERA

It had been observed previously that the iso-agglutinins were largely destroyed by exposure to 60°C. for about 30 minutes. This observation has been confirmed with certain sera, but with others there was very little reduction in titer after such treatment. The other two types of antibody appear to be more thermostable. The sera were stored frozen solid, except in one case (the second serum in Table VI) which was left in the ordinary chamber of the ice box for 3 days. Apart from the fact that the agglutinins do not keep well, the behavior of any given serum is largely unpredictable. Sometimes there appears to be simple deterioration, but with many of the more powerful hyper-immune sera there is a gradual transformation to a blocking type of antibody. In some cases this is partially reversible by heat as shown with the first serum in Table VI. It will also be seen that the enhancing action of serum has become more noticeable. The second serum had kept

well until accidentally left at a higher temperature than usual. The last shows the gradual development of a pro-zone. It is of interest to note that Wiener (13) detected the formation of blocking antibodies on storage; in fact, he first noticed them in stored sera.

From many points of view the behavior of these sera is very inconvenient, but as will be shown below, it may throw light on the significance of the various forms of antibody *in vivo*.

DISCUSSION

It is of interest to compare the situation that obtains here with that occurring with Rh immunization in man. When one considers the taxonomic gulf that separates the two species, the results are surprisingly similar and might well be more so if the dosages given could be made at all comparable. In both cases it appears that a mixture of antibodies is formed, the type of result one obtains on titrating the sera depending upon their relative proportions. A pro-zone of complete inhibition is apparently much more common in mice than in man. This could be due to differences in dosage. The largest pro-zone was seen in a C57 brown animal with a large growth that had persisted for many weeks (Table III). It is difficult to believe that a situation at all comparable has ever occurred in man.

The present writer has been informed by more than one worker in the field that attempts to produce Rh antibodies by the inoculation of volunteers is apt to be disappointing as the titer of the complete antibodies may fall during immunization. This occurs in mice, but here they return again with sufficient dosage. In the experiments reported here this was about equal to the animals' blood volume.

The physical conditions under which cells sensi-

tized with partial antibodies can be made to agglutinate are undoubtedly different in the two species. Nevertheless, in both it appears that these antibodies are not homogeneous. Coombs, Mourant and Race (2) found sera which gave negative results with the blocking test and with Wiener's conglutinin test, but was positive with their antiglobulin test. Similarly, Diamond and Abelson (5) found that about 7 per cent of sera were negative both to direct agglutination and the blocking tests. The addition of the slide test enabled the presence of antibodies to be detected in nearly 100 per cent of cases as does the albumin technic (6). These authors have shown that sera showing no pro-zone and apparently weak agglutinins may have high

TABLE VI: THE EFFECT OF STORAGE ON SERA

Days of storage	Treatment	Titred in	Dilution of serum									
			2	4	8	16	32	64	128	256	512	1024
0	Nil	Saline	—	—	—	—	++	++	—	—	—	—
		50% serum	—	—	—	—	++	+++	+	+	—	—
1	Nil	Saline	—	—	—	—	—	—	—	—	—	—
		50% serum	—	—	—	—	—	—	—	—	—	—
	58.5°/C. for	Saline	—	—	—	tr	tr	tr	tr	—	—	—
	20 minutes	50% serum	—	—	tr	+	++	++	+	tr	—	—
0	Nil	Saline	c	a.c.	+++	++	++	++	++	+	+	tr
		50% serum	c	c	+++	++	++	++	++	++	++	1
28	Nil	Saline	—	—	—	—	—	±	±	±	±	tr
		75% serum	—	—	—	—	—	±	±	±	±	tr
0	Nil	Saline	a.c.	c	+++	+++	+++	+++	+++	+±	±	—
4	Nil	Saline	tr	tr	tr	+++	+++	+++	Not tested			
18	Nil	Saline	—	—	—	+	++	+++	a.c.	+++	Not tested	

titers of partial antibodies (6). It is not clear why some partial antibodies give a strong blocking effect and a pro-zone, whilst others do not. The precise type of titer one gets must be the resultant of the different proportions of all of them. It seems likely that the apparent drop in titer observed in the course of immunization with blood and with C1300 (see Tables I and II) is due to the presence of partial antibodies.

A further complication is introduced by the fact that we are not dealing with pure antigens. The two tumors used here contain at least two isoantigens in common, and it is interesting to note that Dr. Snell has found that in certain crosses C1300 gives two gene ratios. It is striking that all of four A strain tumors (2 studied here and 2 in England) have appeared to contain two antigens. In the case of the growths studied here, it is possible that their proportions are different in each. Anti-sera obtained following immunization with C1300 contain an antibody reacting with C3H cells more frequently than is the case when 15091a is the antigen. This suggests that the former tumor contains more of antigen I, whilst absorption

experiments suggest that 15091a contains more of antigen II. This quantitative difference may explain in part the difference in the antibody response shown by C57 blacks. In man, blocking antibodies are formed more frequently against antigen Rho than against the other Rh antigens.

If one attempts to generalize on the response of mice to hyper-immunization, it is possible to say only that even if the genetic constitution of the recipient is standardized as far as possible, each antigen appears to have its own peculiarities, which again may be varied according to the manner in which it is administered. If we use the same antigen, as was done with 15091a, it is apparent that the genetic constitution of the animal producing the antibody has considerable influence on the type of serum that will be obtained.

In previous publications it was pointed out that a tumor may grow in the presence of antigenic differences between transplant and host. The phenomenon of concomitant immunity made it seem likely that antibodies might be formed under these conditions (9). In the experiments reported here these were demonstrated directly in animals that were certainly going to die from the tumors. This might seem to indicate that antibodies are of minor importance. Such a conclusion is unjustified. Death from bacterial infection may occur in the presence of high titers of antibodies. Furthermore, these animals live much longer than the naturally susceptible mice which do not form iso-antibodies; over 3 months as compared with 3 to 4 weeks. It may well be that other factors than antibodies are of importance in determining the fate of transplanted growths. If the present writer has previously over-emphasised their role (10), it was in order to point out that transplantation immunity is not some novel and mysterious process, but is fundamentally the same as immunity to infection.

It has already been shown that the agglutinins can be absorbed from a serum, leaving the protective action virtually unaltered. It would appear from this that such antibodies are of much the same significance as anti-flagellar bodies in salmonella infections, etc. However, the behavior of the sera on storage suggests another explanation. Even when stored in a frozen state there is a tendency for the agglutinins to be transformed into partial antibodies, of which the blocking antibody appears to be the final product. The mouse stores its antibodies at about 37°C. and it is not unreasonable to suppose that this transformation will be greatly accelerated under these conditions. By analogy with human iso-immunization one might expect the blocking antibody to be of the

greater functional significance. If this is shown to be so, we can draw an analogy between the antibody response and leukocytosis, the "complete" agglutinins corresponding to functionally immature myelocytes. Sera such as those obtained against 15091a being the analogue of an extreme shift to the left.

SUMMARY AND CONCLUSIONS

1. The A strain of mice carries at least 2 antigenic factors in its erythrocytes that are shared by the fixed tissues and are important in transplantation.

2. It has been shown that at least 3 types of iso-antibody may be produced by the mouse: (a) ordinary iso-agglutinins, (b) antibodies that need high concentrations of normal mouse serum to cause agglutination, (c) blocking antibodies, which up to the time of writing can only be recognized by their power to inhibit agglutination.

3. Sera of normal C57 blacks may contain natural iso-antibodies of the second type.

4. The proportions in which these antibodies exist in a given serum varies with the type of tissue inoculated, the interval between inoculations, the interval between inoculation and bleeding and the genetic constitution of the host. Details of the response to inoculation with whole blood and 2 tumors (C1300 and 15091a) will be found in the text.

5. In hyperimmunized animals it is common to find a distinct pro-zone with complete inhibition of agglutination up to a high dilution.

6. Animals dying as a result of tumor inoculation may have very high titers of iso-antibodies.

7. Antibodies needing serum for agglutination are found following a relatively weak stimulus.

8. Such antibodies and blocking antibodies persist longer in the circulation than do ordinary agglutinins.

9. The factor in normal plasma or serum that enhances agglutination is thermostable.

10. On storage in the frozen state there is a tendency for antibodies to be transformed towards the blocking type of antibody.

11. Ordinary agglutinin is without protective function. It is suggested that it may mature *in vivo* to a functional antibody. An analogy is drawn between leukocytosis and iso-antibody formation. Sera with high titers of ordinary agglutinins correspond to an extreme left shift.

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The Uptake of Radiophosphorus in the Phospholipid Fraction of Mouse Epidermis in Methylcholanthrene Carcinogenesis*

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The integration of the chemical, physical, and histological changes in mouse epidermis during carcinogenesis induced by methylcholanthrene has been recently reviewed in a second summarizing report by Cowdry (4). Investigations on the lipids (13, 14), minerals (1), B vitamins (11), and succinic dehydrogenase and cytochrome oxidase (2) have been reported. Experiments are being planned which will enable us to study the uptake of radiophosphorus in the nucleoprotein, acid soluble and phospholipid fractions of mouse epidermis undergoing carcinogenesis by methylcholanthrene. In this paper we wish to describe our investigations on the uptake of P^{32} in the epidermal phospholipid fraction.

EXPERIMENTAL PROCEDURE

The procedures for shaving the mice, applying the carcinogen, and removing the epidermis from dermis have been described (2). In this study methylcholanthrene in benzene, and benzene alone were applied on alternate days for 3, 6, and 12 treatments, and the mice were sacrificed 5 days after the last application of the carcinogen or solvent. For the final stage in our series, the transplantable squamous cell carcinoma of Cooper, Firminger, and Reller was employed (3).

The phospholipid fractions of normal, benzene-treated, and methylcholanthrene-treated epidermis and of the carcinoma were extracted twice with a reflux condenser using about 25 cc. portions of a mixture of 3 volumes of alcohol and 1 volume of reagent grade chloroform. The latter solutions containing the phospholipid were evaporated to dryness on a steam bath, and the lipid was re-extracted with petroleum ether (b.p. 30 to 60°C.) The ether was evaporated on a steam bath, the residues digested in a Pyrex digestion tube with a mixture of 2 cc. of reagent grade nitric acid and 2 cc. of reagent grade perchloric acid. The digests

were made up to a volume of 50 cc., and the orthophosphate content was determined on an aliquot by the method of Truog and Meyer (12) in a Coleman spectrophotometer.

To determine the rapidity of P^{32} uptake in the epidermis, solutions of P^{32} as sodium phosphate were injected subcutaneously into mice. The phospholipid fraction from mice so treated with P^{32} was extracted and digested in the same manner as described above. The perchloric acid solution containing the phosphate was diluted with a few cubic centimeters of water, neutralized with sodium carbonate, and quantitatively transferred to a 25 cc. volumetric flask. One cubic centimeter of the neutralized solution was delivered onto a small watch crystal, and evaporated to dryness in a vacuum dessicator. The resultant samples were mounted in a standard position under a "bell-jar" type of Geiger Mueller counter equipped with a thin aluminum window. Corrections for self-absorbtions were not necessitated because the sample thickness in no case exceeded a few mgm./cm. Decay corrections were made when required by response to a control sample of radioactive phosphate prepared from the same solution used for injection. The total weight of sample was determined in each case by drying the alcohol ether extracted tissue at 105°C. to constant weight. The P^{32} content was then corrected to unit dry weight of lipid free tissue so that all samples were directly comparable as to specific P^{32} content.

RESULTS

The lipid-phosphorus content of normal-untreated, benzene-treated, and of methylcholanthrene-treated epidermis and of the carcinoma is expressed as milligrams of lipid-phosphorus per 100 mgm. of dry fat-free tissue (Table I). This value for untreated epidermis was 0.163 which agreed rather well with that of the epidermis of mice which had received 3 applications of benzene (0.173), and 6 treatments with benzene (0.153). On the other hand, the phosphorus fat-free dry weight value of the epidermis which had been

painted 3 times with the carcinogen was 0.123, a decrease of about 25 per cent from the normal. Those animals receiving 6 and 12 applications of the carcinogen had quantities of 0.120 and 0.112, respectively, which were 26 and 31 per cent less than that of the normal. In the carcinoma, the value increased to 0.311 which was nearly twice that of the normal-untreated epidermis.

TABLE I: LIPID PHOSPHORUS/FAT-FREE DRY WEIGHT RATIO OF MOUSE EPIDERMIS

Number of mice	Number of paintings	Time after first treatment to killing of mice, (days)	Phosphorus/fat-free dry weight ratio
		NORMAL, UNTREATED MICE	
9			0.174
11			0.161
9			0.155
29 (Total)			Average 0.163
		BENZENE-TREATED MICE	
9	3	10	0.184
10	3	10	0.167
10	3	10	0.169
29 (Total)			Average 0.173
11	6	17	0.160
10	6	17	0.168
11	6	17	0.132
32 (Total)			Average 0.153
		METHYLCHOLANTHRENE-TREATED MICE	
8	3	10	0.118
7	3	10	0.120
7	3	10	0.132
22 (Total)			Average 0.123
9	6	17	0.120
9	6	17	0.112
8	6	17	0.128
26 (Total)			Average 0.120
9	12	31	0.115
8	12	31	0.129
9	12	31	0.093
26 (Total)			Average 0.112
		TUMORS	

Tumors from sufficient animals were pooled to give 60-150 mgm. fat-free dry weight samples.

0.321
0.299
0.319
0.303
0.280
0.307
0.340
0.306
0.316
0.322

Average 0.311

To determine the time of maximum uptake of P^{32} in normal-untreated, benzene-treated, and methylcholanthrene-treated epidermis, and of the transplantable squamous cell carcinoma, radiophosphorus as disodium acid phosphate solution was injected subcutaneously (1.0 cc. per mouse), and the mice were sacrificed from 6 to 60 hours after injection of the P^{32}. The results of this experiment, corrected to standard initial activity, are shown in Table II. The time of maximum uptake of P^{32} in benzene-treated, methylcholanthrene-treated epidermis and of the carcinoma was 12 hours, while that of the normal epidermis was at 24 hours. The uptake in the carcinoma was very high, and was maintained higher than the other tissues for 48 hours.

TABLE II: UPTAKE OF P_{32} IN THE PHOSPHOLIPID OF MOUSE EPIDERMIS

Material	Time after injection of P_{32}, (hours)	Counts per minute per 100 mgm. dry fat-free tissue
Normal, untreated epidermis	6	12,100
	12	14,210
	18	15,750
	24	22,640
	36	11,620
Methylcholanthrene-treated epidermis, 3 paintings	12	13,560
	24	13,640
	36	12,520
	48	10,200
Benzene-treated epidermis, 3 paintings	6	11,210
	12	18,000
	18	13,500
	24	11,210
Carcinoma	12	33,620
	24	33,810
	36	27,380
	48	24,330
	60	18,030

In Fig. 1 the specific activity (counts per minute per mgm. lipid-phosphorus) is plotted against the time. The uptake of P^{32} in the benzene-treated epidermis was rapid and maximal at 12 hours and fell quickly, while that of the normal rose slowly reaching its peak at 24 hours, but dropped at about the same rate. On the other hand, the uptake in hyperplastic epidermis and in the carcinoma was rapid and was maintained at near maximal levels for nearly 36 hours. Moreover, the rate of fall of the P^{32} was almost the same for these tissues. The specific activity of the normal epidermis at the time of maximum uptake was somewhat higher than the other tissues, but not significantly so, in view of other data to be shown later in this report.
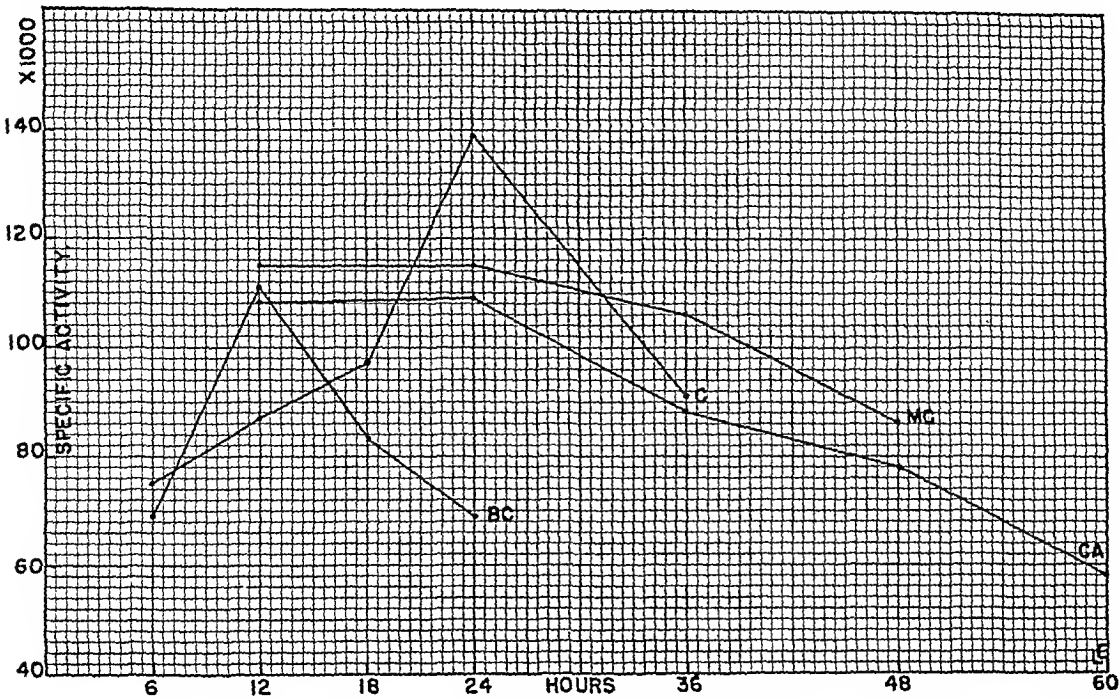
FIG. 1.—Specific activity time curves for benzene-treated (BC), normal-untreated (C), and methylcholan-threne-treated (MC) epidermis, and for the carcinoma (CA).

For the study of the influence of repeated applications of methylcholanthrene in benzene or of benzene alone on the uptake of P^{32} in the phospholipid fraction of the epidermis and of the carcinoma, the time of maximum uptake was that shown in Fig. 1.

In conjunction with the study of the time of maximum uptake of P^{32}, a series of untreated controls and of benzene-treated controls was run at the same time with the samples of P^{32} of nearly the same activity. The results (Table III) expressed as counts per minute per 100 mgm. dry fat-free tissue showed 22,118 counts for normal epidermis, whereas those for mice receiving 3, 3, 6, and 12 applications of benzene were, respectively, 22,075, 24,372, 20,641, and 20,758. Therefore, benzene alone has no appreciable effect upon the uptake of P^{32}.

In another larger experiment with another sample of P^{32}, the uptake of normal, benzene-treated, and methylcholanthrene-treated epidermis was determined (Table IV). The data are expressed as in the preceeding table. The count for untreated controls was 9,500, and for mice treated 3 times with benzene, the value was 9,320. The values for the epidermis which had been painted 3, 6, and 12 times with the carcinogen were, respectively, 8,073, 7,600, and 7,706. In the carcinoma, the uptake rose to 20,450 counts per minute. These

TABLE III: UPTAKE OF P^{32} IN THE PHOSPHOLIPID FRACTION OF MOUSE EPIDERMIS

Number of mice	Number of paintings	Time after first treatment to killing of mice,	Counts per minute per 100 mgms. fat-free epidermis	Corrected to standard initial activity
		(days)		
		NORMAL, UNTREATED MICE		
5			1,064	
5			1,081	
5			926	
15 (Total)			Average 1,024	22,118
		BENZENE-TREATED MICE		
5	3	10	959	
5	3	10	1,009	
5	3	10	1,097	
15 (Total)			Average 1,022	22,075
5	3	10	1,976	
5	3	10	2,261	
5	3	10	1,856	
15 (Total)			Average 2,031	24,372
5	6	17	875	
5	6	17	·785	
5	6	17	929	
15 (Total)			Average 863	20,641
5	12	31	988	
5	12	31	869	
5	12	31	1,025	
15 (Total)			Average 961	20,758

counts are considerably lower than those given in Table III, which is due to the fact that a solution of radiophosphorus of different specific activity was administered. No attempt was made to inject the same amount of P^{32} per gram body weight since the activity of our solutions varied too much for this purpose. We chose to compare the specific

TABLE IV: UPTAKE OF P^{32} IN THE PHOSPHOLIPID FRACTION OF MOUSE EPIDERMIS

Number of mice	Number of paintings	Time after first treatment to killing of mice. (days)	Counts per minute per 100 mgms fat-free epidermis	Corrected to standard initial activity
		NORMAL, UNTREATED MICE		
8			2,909	
8			3,038	
9			2,931	9,500
—			——	
25 (Total)			Average 2,959	
		BENZENE-TREATED MICE		
5	3	10	2,493	
5	3	10	3,074	
5	3	10	2,153	9,320
—			——	
15 (Total)			Average 2,573	
		METHYCHOLANTHRENE-TREATED MICE		
5	3	10	7,816	
6	3	10	8,143	
6	3	10	8,261	8,073
—			——	
17 (Total)			Average 8,073	
5	6	17	2,300	
5	6	17	2,292	
5	6	17	2,848	8,000
4	6	17	2,475	
5	6	17	3,225	
4	6	17	2,575	7,200
—			——	
28 (Total)			Average 2,619	7,600
5	12	31	6,811	
5	12	31	7,157	
5	12	31	8,071	
5	12	31	8,785	7,706
—			——	
20 (Total)			Average 7,706	
		TUMORS		
			15,305	20,500
			15,217	20,000
Tumors from sufficient animals were pooled to give 60-150 mgm. fat-free weight per sample			15,138	19,900
			14,303	18,700
			17,333	22,600
			16,033	21,000
			——	——
			Average 15,555	20,450

activities in the different experiments. The specific activity of the epidermal lipid phosphorus of the mice shown in Table IV did not differ significantly (Table V). The specific activity of normal, and benzene-treated epidermis was respectively 58,282, and 60,915, while the respective values for the

TABLE V: RATIO OF COUNTS PER MINUTE PER 100 MGM. DRY FAT-FREE TISSUE AND MGM. LIPID PHOSPHORUS ON THE SAME BASIS OF REFERENCE (SPECIFIC ACTIVITY)

Material	a Counts per minute per 100 mgm. dry weight	b Mgm. lipid phosphorus per 100 mgm. dry weight	$\frac{a}{b}$ (specific activity)
Normal, untreated epidermis	9,500	0.163	58,282
Benezene-treated epidermis, 6 paintings	9,320	0.153	60,915
Methylcholanthrene-treated, 3, 6, and 12 paintings	7,745	0.118	65,635
Carcinoma	20,450	0.311	65,755

hyperplastic epidermis and carcinoma were 65,635, and 65,755. The specific activity values from Fig. 1 at maximal uptake time in the normal and treated epidermis and in the carcinoma are not significantly different, and calculated ratios for the normal and benzene-treated epidermis of mice shown in Table III would be *quite constant*. Although the uptake varies somewhat in the different experiments, the specific activities are quite constant, which demonstrates no appreciable significance in the phospholipid turnover of the differently treated epidermises, and of the carcinoma studied here.

DISCUSSION

In an investigation of the rate of phospholipid turnover in 4 types of transplantable tumors in mice (a mammary carcinoma, a lymphoma, a lymphoblastoma, and sarcoma 180), Jones, Chaikoff, and Lawrence (6, 7) observed that the phospholipid turnover resembled that in tissues such as liver, kidney, and intestine, rather than in tissues like muscle or brain, which are less capable of regeneration and growth. They also observed that the rate of turnover for the types of tumors was not uniform, but that each displayed a characteristic activity. In another study, the same authors demonstrated that the total phosphorus turnover of three neoplastic tissues (a mammary carcinoma, a lymphoma, and a lymphosarcoma) showed a high and rapid uptake of P^{32} in the early intervals after its administration, and that the malignant tumors had a pronounced capacity for retaining P^{32} for a long time in contrast to normal tissues with equal levels of P^{32} (8). In our study on epidermal carcinogenesis, the specific activity time curves showed no appreciable difference at maximum uptake time between normal benzene-treated, and methylcholanthrene-treated epidermis and the carcinoma. However, the specific activity time curves of the carcinoma and hyperplastic epidermis, the latter being chemically precancerous (2), was quite similar in that both re-

tained their radiophosphorus at a higher level for a longer period of time, and decreased at nearly the same rate.

From experiments on the rate of turnover by lecithins and cephalins of rat carcinosarcoma 256, Haven (5) concluded that the rapid turnover of the lecithins indicated participation of lecithins in metabolic activities. The slower rate of turnover by cephalins suggested relationship to cell structure. Marshak (9) has made a detailed investigation of the uptake of P^{32} by the nuclei of liver and tumor cells, and has shown, among other things, that the nuclei of tumor cells accumulate more P^{32} than do normal liver nuclei. This may be due to greater mitotic activity of the tumor cells and not to the possession by them of a different and characteristic type of metabolism (9). Investigations on tumors employing labelled phosphorus have dealt chiefly with the leukemias (references cited by Scott [10]) which are obviously quite different from carcinomas in our experiments.

SUMMARY

The effect of methylcholanthrene on lipid phosphorus and on the uptake of phosphorus in the lipid fraction of mouse epidermis undergoing carcinogenesis induced by methylcholanthrene was investigated by the use of P^{32}. The carcinogen caused a drop of 30 per cent in the lipid phosphorus content of the epidermis, but in the carcinoma, the lipid phosphorus content was nearly twice that of the normal. The specific activity time curves of normal, benzene-treated, and methylcholanthrene-treated epidermis and of the carcinoma showed no appreciable difference in the specific activities at the time of maximum P^{32} uptake. The rate of uptake in the hyperplastic epidermis and in the carcinoma was rapid, these tissues retained their labelled phosphorus for a considerable time, and both showed a similar fall in activity. The uptake in benzene-treated epidermis was at a maximum at 12 hours, while that of the normal was slower and at a maximum at 24 hours. The rate of fall in each was quite rapid and similar. Other experiments demonstrated no significant difference in the specific activities of normal epidermis or of epidermis treated 3, 6, and 12 times with benzene alone or with the carcinogen in benzene on alternate days during 10, 20, and 30 days. Although the carcinoma had a high and rapid uptake of P^{32}, its specific activity was similar to that of the treated epidermis.

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The Carcinogenic Activity of 2-Acetaminofluorene
Characteristics of the Lesions in Albino Rats*

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Attention has been called to the development of a variety of tumors in rats and mice after the administration of 2-acetaminofluorene by mouth (2, 11). Species differences in susceptibility and in distribution of proliferative lesions have been noted (1, 12), but animals of a single strain have shown fairly consistent development of characteristic lesions. We have used principally albino rats originally of the Stanford strain (11), so the discussion to follow will deal with tumors in these highly susceptible animals.

It is probable that the tumors are induced not by the acetaminofluorene itself, but by some derived product, possibly aminofluorene (13). The nature of the resultant tumors is similar whether acetaminofluorene or aminofluorene is administered to rats.

Focal proliferation of a number of different types of epithelium has appeared following continued feeding of acetaminofluorene in concentrations in the diet ranging from 0.5 per cent to 0.004 per cent. The other characteristics of the diet have been described previously (11). It is not necessary to continue the administration of the substance until the time of appearance of lesions; a period of administration as short as 25 days may be followed months later by the development of tumors (12).

Since the first report further observations have resulted in the detection of a few additional types of lesions and have given more information concerning the frequency of proliferative changes. Tissues from 108 rats which had lesions apparently due to acetaminofluorene have been studied histologically. The animals died or were killed at various intervals after administration of the substance in different quantities, so that the circumstances under which the tumors developed are not closely comparable. Since some of the animals, including all that were fed more than 0.125 per cent acetaminofluorene in the diet, were killed or died early in the course of an experiment before advanced lesions appeared, it was decided to include in this report only those rats which, because of the appearance of multiple lesions, had presumably received a thoroughly effective exposure to the compound. There were 84 such animals, 22 males and 62 females, and in most of them opportunity for the development of multiple lesions was great because they lived until their condition was poor or until large external lesions had appeared. The period of observation ranged from 104 to 695 days following the institution of the augmented diet, which contained from 0.008 per cent to 0.125 per cent acetaminofluorene for intervals not always as long as the observation periods.

TABLE I: INCIDENCE OF THE MOST COMMON LOCAL HYPERPLASTIC AND NEOPLASTIC LESIONS IN 84 RATS (22 MALE AND 62 FEMALE) IN WHICH TWO OR MORE TISSUES WERE INVOLVED

Organ	Number of animals with lesions Male	Number of animals with lesions Female	Total incidence, %	Proportion malignant, %
Liver	21	57	93	22
Bladder	16	51	80	21
Lung	8	24	38	16
Head	5	21	31	92
Breast	3 #	23 #	31	31
Kidney	3	17*	24	25
Uterus		13†	21	8
Thyroid	2	9	13	0
Gastrointestinal tract	1	4	6	60

\# Includes one subcutaneous fibroma.
*Includes one carcinoma of the upper ureter.
†Includes one sarcoma.

Table I indicates the incidence and distribution of hyperplastic changes in the 84 rats that had more than one type of lesion. Since the acetaminofluorene was administered in different dosages in different animals, the indicated incidence of the various lesions is of value principally in showing the relative frequency of the different changes. After autopsy and fixation of the tissues in 4 per cent formaldehyde, histological sections stained with hematoxylin and eosin were prepared from all grossly abnormal tissues except for 2 mammary

tumors, which were lost. Many organs without gross lesions were also sectioned. In all there were sections from the liver of 83 animals, the urinary bladder of 79, the lung of 78, the kidney of 77, the pancreas from 76, the thyroid gland from 60, the ovary from 37, and the uterus from 32 animals. Numerous sections of heart, spleen, testis, stomach, intestine, thymus, hypophysis, salivary gland, skin and bone were examined histologically but they showed no proliferative lesions in addition to those identified by gross examination. Thirty-nine brains examined histologically have shown no lesions suggesting that described as a glioma by Lopez (7), and no other abnormalities have been found in them.

There was much variety in the degree of change in the affected organs of different animals, ranging from focal proliferative lesions which were visible only microscopically, to large grossly recognizable nodules which greatly distorted the shape of the involved organs. Multiple nodules in the liver commonly increased its size by more than 100 per cent. The lesions have not been classified according to size and no distinction between nodular hyperplasia and adenoma formation has been made because no basis has been found for anatomical separation of these states among the experimental lesions. However, an attempt has been made to classify lesions as malignant if there was infiltration of abnormal cells among pre-existing tissue elements. In cases where there was only questionable slight hyperplasia the tissue has been listed as unaffected, and no lesions are recorded as malignant unless distinct infiltration of adjacent tissues could be recognized histologically. It is likely that some instances of malignancy were not detected by this method. In general, the extent of the hyperplasia was great and cell differentiation was poor when infiltration of tumor cells was present, but there were several exceptions. Most of the tumors were composed of epithelial cells, although 5 animals had leukemia and 2 had sarcomas. One of the latter arose in the muscles of the leg and the other developed in the uterus.

The following paragraphs present the types of tissue change that have occurred with sufficient frequency to suggest that they are specific effects of the administration of acetaminofluorene.

Liver nodules.—These were the commonest and most prominent lesions, causing gross deformity of the liver in more than half of the animals. The lesions were always multiple and some reached a diameter of 3 or 4 cm. Most of the nodules were composed of cords of hepatic cells which were similar to, but distinctly different from the normal liver cells (Fig. 1). The cords were sometimes irregular and did not form a lobular pattern, although they bordered prominent sinusoidal spaces. No portal connective tissue spaces could be seen within the nodules. The cytoplasm of the abnormal cells had a varied appearance. In some parts it was more dense and more uniformly stained than normal, but in others it had a reticulated appearance or was distinctly vacuolated. Its volume per cell was distinctly greater than normal. The nuclei were large and quite varied in size and intensity of staining. Nucleoli were large and sometimes multiple. Mitotic figures were prominent in some of the larger nodules. Bordering the sinusoids there were occasional prominent endothelial cells resembling Kupffer cells. In a few places these contained a little light brown pigment that did not give the Prussian blue reaction for iron. There was no evidence that the abnormal nodules of hepatic cells were drained by bile ducts, yet most of them were not distinctly jaundiced. Several of the larger nodules, however, were grossly yellowish and they showed scattered small deposits of iron-free yellow pigment not only in the endothelial cells, but also in some of the hepatic cells.

In many of the livers there were additional nodules of quite different appearance. These were composed of small duct-like structures and cysts which had a lining of simple low columnar or flattened epithelium (Fig. 2). Separating these structures was a little dense fibrous tissue. Certain of the cysts were multilocular and among them in a few places were groups of hepatic cells which appeared to have been isolated from pre-existing

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DESCRIPTION OF FIGURES 1 TO 6

Fig. 1.—Small nodule of hyperplastic hepatic cells adjoining normal liver tissue. At the edge of the nodule on the right is a central vein. Mag. × 120.

Fig. 2.—Nodule in liver composed entirely of cysts lined by low columnar epithelium. Mag. × 48.

Fig. 3.—Lung nodule formed by proliferated epithelial cells within alveoli. This is apparently benign. Mag. × 48.

Fig. 4.—Similar lung tumor. Mag. × 120. Clumps of cells in alveolar lumen can be distinguished from layer of alveolar lining cells.

Fig. 5.—Epithelial tumor arising from renal pelvis. Narrow rim of atrophic kidney tissue borders it on the left. Mag. × 48.

Fig. 6.—Border of a malignant tumor of squamous epithelium arising adjacent to the deep end of the external auditory canal. Mag. × 120. Projections of tumor cells have infiltrated skeletal muscle.
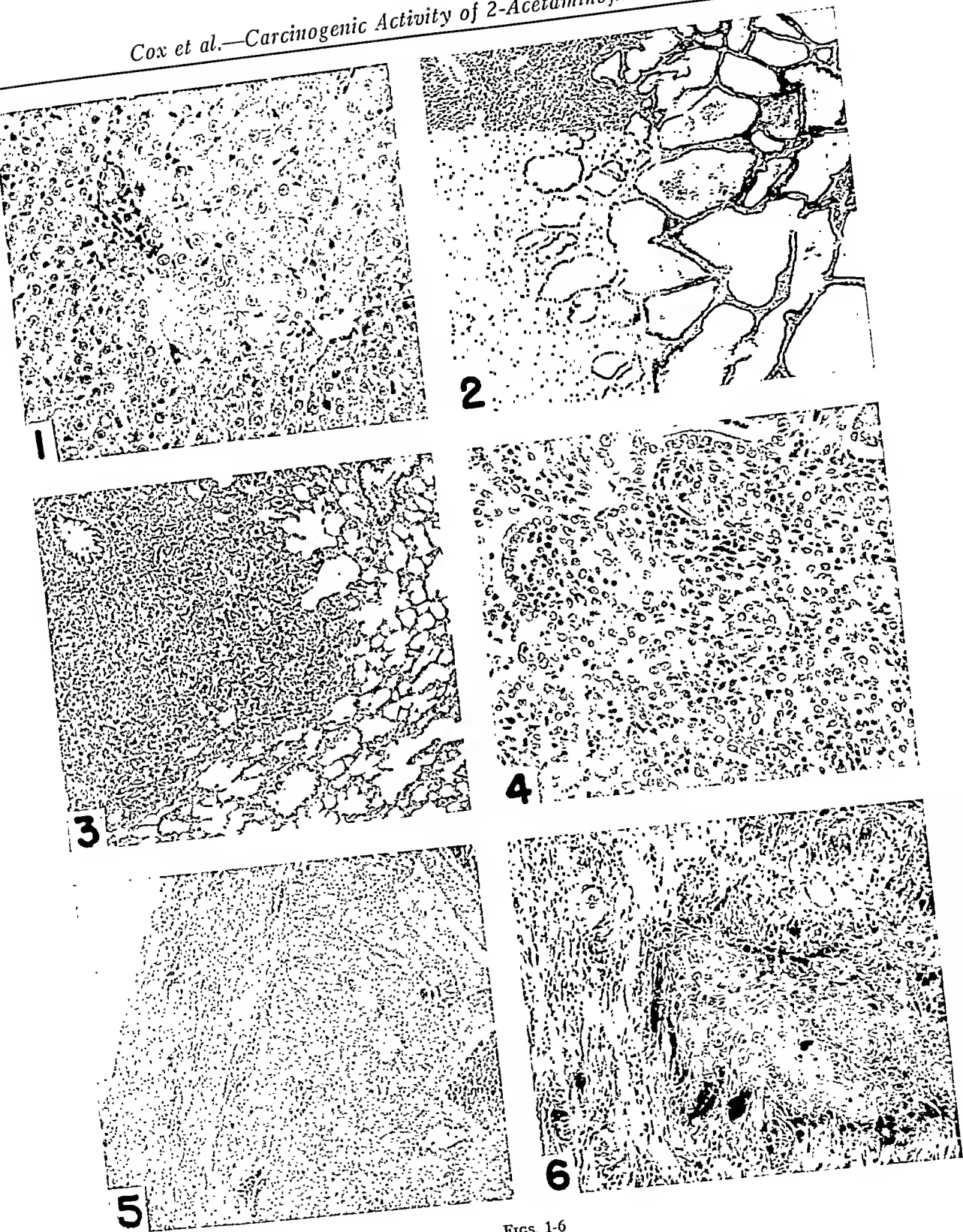
Figs. 1-6

liver tissue. The cells forming these cysts were more uniform than those of many of the solid nodules.

Frequently the same liver contained nodules of different types, and when one nodule showed malignant changes, others appeared benign. In the presence of large tumors, other parts of the liver regularly contained microscopic groups of enlarged cells forming discrete but non-encapsulated nodules. In some of the nodules of hepatic cells there were numerous small spaces often reaching a diameter of 1 mm. These were bordered by flattened cells and produced an appearance suggesting abnormal dilated bile ducts. These duct-like structures appeared to be integral parts of the hyperplastic nodules, although the manner of their development was not determined. These nodules containing hepatic cords and glandular structures correspond to the adenohepatomas described by Opie (8) after feeding *p*-dimethylaminoazobenzene. Two of the malignant liver tumors contained duct-like structures associated with other cells resembling hepatic cells.

Several of the large partially necrotic lesions were accompanied by infiltration of a variety of inflammatory cells but this was not observed in small lesions which were likewise unassociated with any fibrosis of the liver substance.

Nodular proliferation of epithelium in the lungs.— In lungs from 22 rats there was a characteristic sort of focal epithelial proliferation similar to that which has been observed in mice by many investigators. In 17 animals multiple lesions were found. A number were demonstrable only histologically, and since from most animals only one or two sections of lung tissue were prepared, it is certain that other undisclosed small lesions were present. Inflammation was prominent in some lungs and it is possible that instances of nodular epithelial proliferation were concealed by it. However, many of the nodules were not associated with inflammation. The lungs from control animals have not been examined by serial sectioning, but in numerous sections from old animals which did not receive acetaminofluorene, no lesions of this type have been seen.

Most of the pulmonary nodules were less than 4 mm. in diameter. They were localized though not encapsulated, and their appearance suggested a benign proliferative process. The principal constituents were abnormal epithelial cells within the alveolar spaces (Figs. 3 and 4). The pulmonary structure persisted within the nodules but the alveolar spaces were smaller than those elsewhere. Each alveolus in the altered region was lined by a layer of simple cuboidal or low columnar cells and

in most of the spaces there were also small solid masses of polygonal, similarly stained cells. A small cleft usually separated these from the layer bordering the alveolar wall. The proliferated cells had nearly round nuclei with prominent small chromatin particles but no large nucleoli. Scattered nuclei were hyperchromatic and there were a few mitotic figures. The cells commonly appeared columnar on one side of an alveolus and cuboidal or even flattened on another. In a few places, clumps of 4 or 5 nuclei were very closely placed, suggesting formation of small syncytial masses. Most of the cells had scanty, slightly acidophilic cytoplasm. The appearance suggests that these may have been derivatives of normal alveolar lining cells. Small bronchi were present within some of the nodules but their epithelium was distinctly different from that of the intra-alveolar cells. The lesions in our rats were similar to those induced in mice with dibenzanthracene and methylcholanthrene by Grady and Stewart (5), who have presented satisfactory evidence based upon the study of serial sections, that the proliferating cells of their tumors originated in the alveoli.

The lungs from 5 additional acetaminofluorene-treated rats contained larger masses composed of less uniform cells which had destroyed lung tissue and had penetrated through the pleura or into large bronchi. The cells of these malignant tumors were small, they were arranged in small irregular clumps and strands, and their appearance suggested an epithelial origin. In places they showed a resemblance to the cells of the benign lesions. A lung from one additional animal showed a small papillary intrabronchial benign tumor in which the epithelium was similar to that of the bronchial wall.

It was suggested in our original report that the metaplastic squamous epithelium lining bronchi in certain regions of chronic inflammation in some lungs might be a specific effect of acetaminofluorene feeding. Similar regions of metaplasia have been found in additional animals, but they have also appeared in a number of old rats with chronic pulmonary inflammation which did not receive any carcinogenic substance and which showed no other lesions like those in the acetaminofluorene-treated animals. Therefore, this bronchial epithelial metaplasia cannot be regarded as a specific effect.

Epithelial proliferation in the urinary tract.— With the exception of a benign tubular adenoma in one kidney, all of these lesions have been derived from transitional epithelium of the bladder, renal pelvis and ureter. The lesions were always focal

and were usually multiple. There was wide variation in their size as well as their character. Small lesions sometimes consisted of no more than slight local thickening. The cells in the thickened zones were usually enlarged and arranged less regularly than in normal transitional epithelium. In many instances they resembled stratified squamous epithelium and some showed various degrees of keratinization. Most of the nodules were flat but elevated papillary lesions were common. Malignancy was recognized by deep penetration of irregular cords of tumor cells, which were usually less differentiated than the cells of the benign lesions. They frequently resembled stratified squamous epithelium rather than transitional epithelium. Some surrounded small foci of keratinization.

The hyperplastic changes were much more frequent and more prominent in the bladder than in the remainder of the urinary tract. Several instances previously reported as hyperplasia of the kidney pelvis are omitted from the present classification since they were minimal. Three carcinomas of the renal pelvis were present, although infiltration of adjacent tissue was not extensive. All these tumors were papillary in some parts; 1 was composed of keratinizing squamous epithelium, whereas in 2 the tumor cells resembled transitional epithelium (Fig. 5). A single tumor in the region of the upper end of one ureter was a squamous cell carcinoma. Since routine histological examination of the ureters was not made, no knowledge of the incidence of small ureteral lesions is available.

Subcutaneous tumors of the side of the head.— These tumors all appeared just anterior to the ear and were covered by intact movable skin when they were small. They were composed largely of squamous cells in abnormal arrangement. Usually the central part contained a cavity filled with desquamated keratinized material. The wall was formed by irregular masses of stratified squamous epithelial cells of varied size and staining reaction. These were supported by thin fibrous strands and frequently surrounded small masses of keratin. At the periphery in all but two instances there was infiltration of adjacent structures by tumor cells (Fig. 6), and the appearance was characteristic of squamous cell carcinoma. The two apparently benign lesions were papillary tumors nearly 1 cm. in diameter lying within cysts which occupied the same position as the infiltrating tumors.[1] The cyst walls were lined by strati-

fied squamous epithelium which had a smooth outer surface except for some blunt rounded projections of epithelium and a number of protruding lobules of sebaceous gland tissue in one place. The papillary portion of these tumors protruded into the cyst cavity and was composed of long thin folds covered by keratinizing stratified squamous epithelium. Parts of the cyst were packed with desquamated keratinized material.

The origin of all of the tumors of the head was apparently the same. They all developed in the same location and had a similar appearance. Although they sometimes perforated the auditory canal and were always closely attached to it, the lining of the canal was intact over several tumors except for a single small round hole in each case. The largest of these openings was 2 mm. in diameter; each had a smooth border and led into a cavity within the tumor. These openings suggested dilated duct orifices and together with the other features of these tumors, pointed to a probable origin from some adjacent accessory structure, rather than from the lining of the auditory canal. The most likely origin is the sebaceous glands which are normally prominent adjacent to the auditory canal in this region. The skin of the head was freely movable over the smaller lesions and can be eliminated as the point of origin even though the large tumors frequently produced ulceration of the skin. Parotid gland tissue was present adjacent to some of the tumors but there was a separating layer of fibrous tissue and the appearance did not suggest an origin from this tissue.

Tumors of the breast.—In our original report the mammary origin of several subcutaneous glandular tumors was not established with certainty, although it was suggested as the most likely possibility. In the animals studied subsequently, it has been possible to identify mammary tissue immediately adjacent to the tumors and there have been several instances of irregular hyperplasia of mammary tissue without distinct tumor formation (Fig. 7). The structural similarity of some of the subcutaneous tumors to portions of the hyperplastic mammary tissue provides further evidence that the tumors were of mammary origin.

In the hyperplastic glands there were usually irregular groups of mammary ducts, frequently somewhat dilated, and among some of them there was more than the usual amount of fibrous tissue, showing mild lymphocytic infiltration. Frequently there were prominent lobules of small acini. Thirty-two grossly visible tumors developed in 26 animals. Most of these were composed of irregular small glandular structures resembling mammary

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[1] In several additional animals killed when tumors of this type were small, similar apparently benign lesions have been found.

ducts, lined by cuboidal or pseudostratified epithelium and scattered through different amounts of fibrous stroma. Some contained groups of structures resembling acini (Fig. 8). In some the connective tissue was prominent, as in fibroadenomas, but since there was no sharp dividing line among 19 of these benign tumors, they have all been classed as adenomas. One of the smaller lesions lay in a cyst-like space about 1 cm. in diameter lined by low columnar epithelium which was frequently piled up to a layer several cells thick. The intracystic nodule was 3 mm. broad and was attached on one side. It was composed of small irregular glands in a little cellular stroma.

Nine additional tumors were classed as malignant. These showed less uniform glandular structures than did the benign tumors, and there were commonly thin cords of atypical epithelial cells without demonstrable lumen. Two of the tumors contained cystic spaces lined by columnar cells and enclosing protruding masses of glandular tumor tissue, suggesting pre-existing papillary adenomas.

Two benign subcutaneous tumors were composed only of fibrous tissue without any glands. The origin of these is not certain, although their location was similar to that of the glandular tumors, and bordering the one which appeared in a male rat was a layer of mammary tissue. Two other subcutaneous tumors apparently of mammary origin were not examined histologically because the tissue was lost. They have been classified as benign lesions.

Nearly all the subcutaneous tumors developed in female rats, but 3 appeared in males. None of the tumors in the males has been regarded as malignant.

These observations indicate that the mammary gland in acetaminofluorene-treated rats undergoes changes comparable to those of other organs showing nodular hyperplasia in association with, and presumably preceding, tumor formation. The frequency of this hyperplasia is not known, since the mammary glands were not subjected to routine histological study.

Nodular proliferation of endometrial glands.— Eight uteri from the experimental animals showed local enlargements up to a diameter of 5 mm., and of 32 uteri which were examined histologically, 13 showed irregularities of the endometrium. These lesions all appeared in animals surviving for more than 250 days following the onset of treatment; most of the animals were more than 400 days old. It is possible that ageing played a more important part in the development of these changes than in most of the others observed in this group of animals, although comparable alterations have not been seen in other old rats.

Some of the glands in the altered zones resembled normal endometrial glands. They were small, fairly uniform, and lined by a single layer of cuboidal or low columnar epithelium. There was an accompanying increase in endometrial stroma, which sometimes contained a few scattered lymphocytes and brown pigment-filled macrophages, suggesting that bleeding had occurred. Not infrequently some glands were dilated to a diameter of more than 1 mm. These cyst-like spaces contained a small amount of eosinophilic coagulum and their lining epithelium was usually flattened. The proliferation was eccentric with respect to the lumen and the myometrium was expanded over the surface of the proliferated nodules (Fig. 9). There was no evidence of malignancy. The lower portion of one uterus was destroyed by a large sarcoma composed chiefly of spindle-shaped cells. It apparently arose in this organ.

Thyroid gland nodules.—In our first animals no proliferative nodules were noted in the thyroid gland. Bielschowsky (3) has stated that acetaminofluorene alone does not evoke thyroid hyperplasia. However, in 11 of the animals more recently studied and included in the present series there was distinct nodular irregularity of the histological structure of this gland. A possible factor of importance is that these animals were older at death than most of the animals studied previously. In four glands the nodular appearance was due principally to variation in size of follicles and in amount of colloid contained. There were several distinct but not encapsulated nodules up to 1 mm. in diameter (Fig. 10). Among abnormally large colloid-filled follicles in the nodules there were usually small empty follicles. The proliferative nature of the process is evidenced by epithelial cells of normal or even increased height forming the large follicles. In five adenoma-like nodules much of the epithelium was composed of tall

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DESCRIPTION OF FIGURES 7 TO 10

FIG. 7.—Irregular hyperplasia of mammary tissue without formation of distinct tumor. Mag. × 48.

FIG. 8.—Benign fibroadenoma of mammary glands, remnant of which appears above the tumor. Mag. × 120.

FIG. 9.—Nodule of hyperplastic endometrium. This presents no evidence of malignant growth. Mag. × 48.

FIG. 10.—Nodule in thyroid gland formed by large follicles with abnormally tall lining epithelial cells. Mag. × 120.
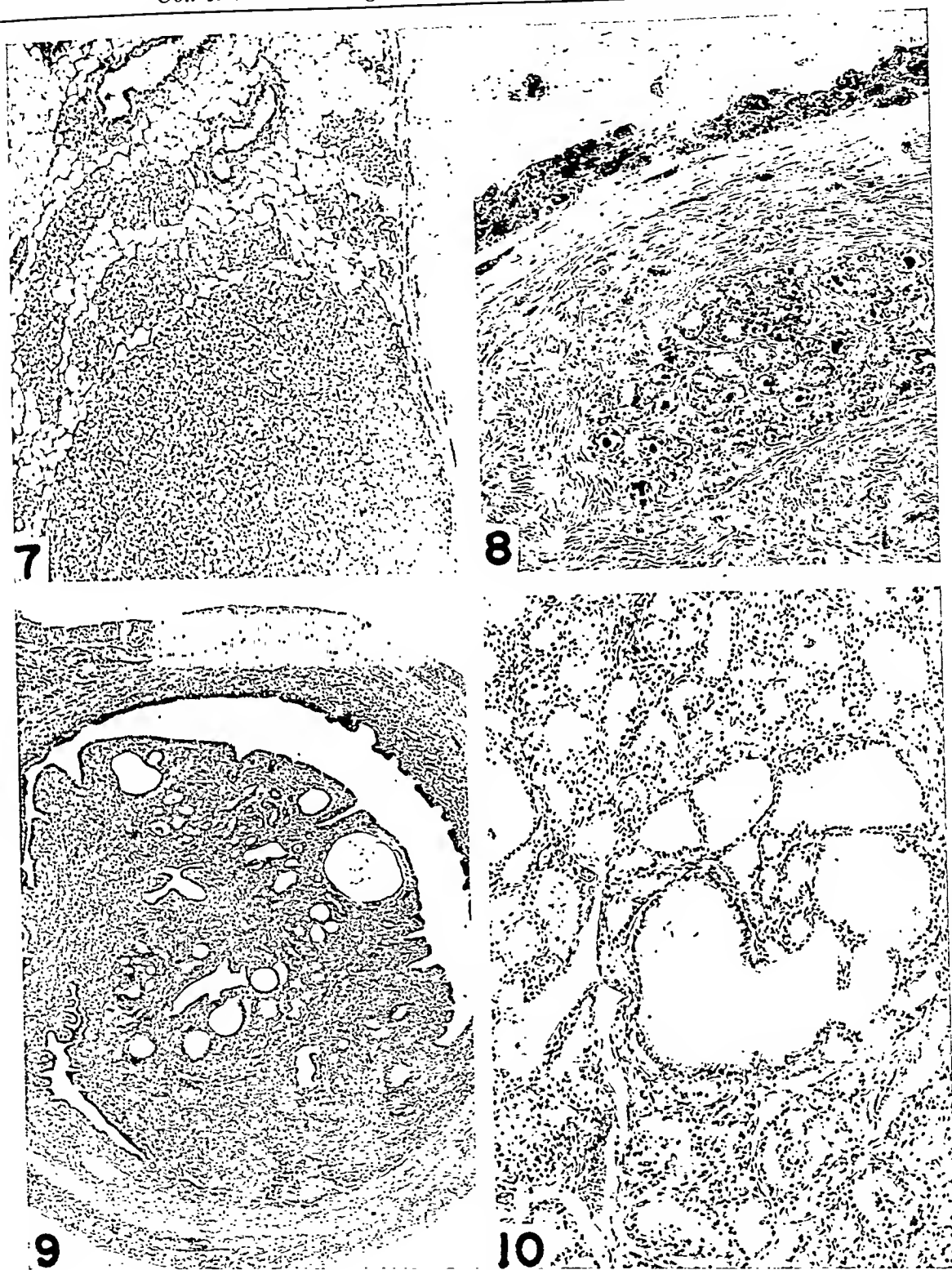
FIGS. 7-10

columnar cells. It formed projecting folds into the lumen of moderately large collapsed follicles which contained very little colloid. The structure in these nodules had little resemblance to the normal thyroid, but their origin in this organ seems certain. Several lay deep in the substance of the thyroid gland and none were situated upon the surface as would be expected if they were derived from the parathyroid glands. These in some sections were demonstrable as separate structures. Tumors of parathyroid glands, which have been reported after acetaminofluorene administration by Heiman and Meisel (6), have not been recognized in our animals.

Tumors of the stomach and intestine.—One small adenocarcinoma of the stomach was situated beneath a shallow ulcer 2 mm. broad in the pyloric zone where a mass of atypical small glands extended into the submucosa (Fig. 11). There was also heavy infiltration of small mononuclear cells. There was not much variation of the tumor cells but the structures formed did not closely resemble normal mucosal glands. They reached the muscularis but did not penetrate it. The tumor cells contained a number of mitotic figures. Another glandular tumor of the submucosa of the stomach has been classed as an adenoma because the tubular structures forming it were more uniform than those of the tumor described above and it formed a well circumscribed, though not encapsulated, nodule.

A somewhat similar tumor was situated in the ileum of another animal. It lay principally in the submucosa and elevated the mucosa, which was not sharply distinguishable from the tumor in some places. Most of the tumor cells contained vacuoles and at the deep margin there were several glands containing Paneth cells. Although this tumor reached the muscularis, it had a sharp border and has been classified as benign.

The colons in 2 animals contained extensively infiltrating adenocarcinomas composed of moderately irregular, coarse, glandular structures with a lining of columnar epithelium. These tumor cells penetrated the entire thickness of the intestinal wall (Fig. 12). In one of these animals there were multiple metastatic growths.

Changes in the pancreas.—In our original report,

microscopic nodules of prominent pancreatic acini were described in more than half of the animals in which the pancreas was examined histologically. There was also one carcinoma apparently of pancreatic origin. These lesions have been seen much less frequently in subsequent animals and no further instances of tumor of this organ have been found. There appears to be little doubt of the proliferative character of this change when the lesions are marked, but review of the sections from the original series throws doubt upon the significance of those changes reported as slight. Further observations are necessary to determine the importance of the pancreatic changes. In an accessory lacrymal gland of one rat a tiny sharply defined group of slightly enlarged acini resembled the pancreatic lesions. In several others clusters of tiny cystic spaces were seen in the pancreas, but these were not numerous and it is not possible at present to relate them to the acetaminofluorene feeding.

Leukemia.—Five of the animals had leukemia, with infiltration of multiple organs. In each case the liver and spleen were involved. Leukemic cells were present in all parts of the liver lobules, and many cells lay within the sinusoids. The appearance of these abnormal cells suggested that they were myelogenous, although studies of the cells in smears have not been made. The incidence of leukemia in the acetaminofluorene-treated animals is considerably higher than that in control animals of our colony, but leukemia has been observed frequently under other experimental conditions (13), and therefore the relation of leukemia to the acetaminofluorene feeding is less distinct than in the case of most of the other lesions described above.

Miscellaneous proliferative lesions.—A sarcoma situated among the leg muscles was described in our original report. The other sarcoma that we observed is discussed in conjunction with the uterine changes. The development of only 2 sarcomas in contrast to 76 malignant epithelial lesions emphasizes the relative rarity of tumors in the connective tissue of animals fed this agent. A malignant tumor in the mediastinum of one animal infiltrated the pericardium extensively to form a firm white layer 2 to 3 mm. thick completely en-

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DESCRIPTION OF FIGURES 11 TO 14

Fig. 11.—Infiltrating adenocarcinoma at base of ulcer in stomach. Mag. × 120.

Fig. 12.—Adenocarcinoma of colon which has penetrated all layers of the intestinal wall (right). A piece of unaffected mucosa is seen at left. Mag. × 48.

Fig. 13.—Metastatic nodule in lung from malignant liver tumor. Metastatic cells are arranged like those of the primary lesion. Mag. × 120. Two arteries and a bronchus are shown.

Fig. 14.—Metastasis in the omentum of adenocarcinoma from lesion of colon illustrated in Fig. 12. Mag. × 120.
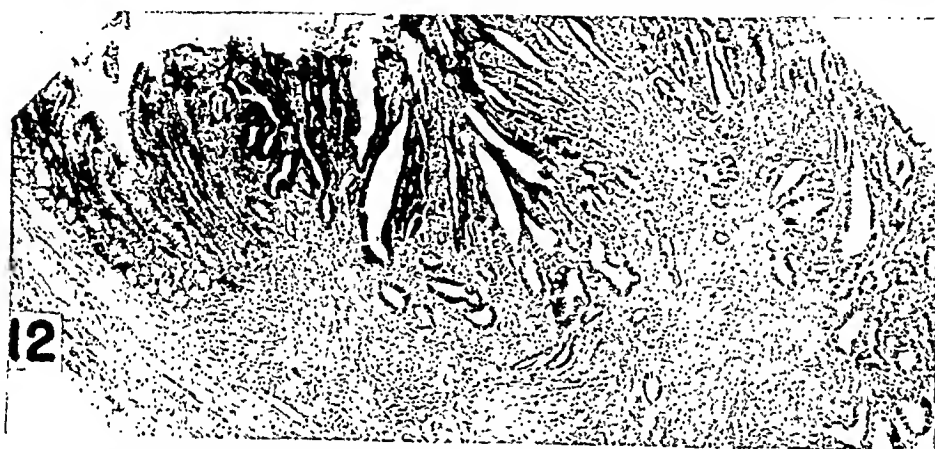
FIGS. 11-14

casing the heart. The tumor cells were polygonal in places, but elsewhere they were elongated and spindle-shaped. Around a number of small necrotic foci the cells were larger and had a palisade arrangement. Similar palisading of the cells occurred at the surface of the epicardium. The cells were not uniform, many contained mitotic figures, and groups of them frequently lay within small vessels bordering the myocardium. It is suggested that this is a tumor of the thymus, although proof of its origin has not been obtained.

Metastasis from malignant tumors.—Metastatic lesions were found in 13 of the 52 animals bearing carcinomas. These secondary tumors were usually multiple but were of only 2 types. No animal showed metastasis from more than 1 tumor. In 11 instances metastasis occurred from liver tumors. The secondary nodules, which were structurally like the hepatic cell tumors in the liver, were found in the lungs (Fig. 13) of 8 rats, in the peritoneum and adjacent tissues of 4, and in the lymph nodes of 1 animal. No metastatic nodule contained any cyst-like structures. The other type of metastasizing tumor was adenocarcinoma (Fig. 14). Secondary growths of this type occurred in only 2 animals and were limited to tissues covered by peritoneum. In one the primary tumor arose in the colon and in the other the primary tumor was not found.

None of the tumors of the breast, urinary tract, lung or head, and neither of the 2 sarcomas, had spread to distant sites, although in several instances small satellite tumor nodules were seen adjacent to a primary tumor.

DISCUSSION

The present discussion of tumors resulting from the feeding of acetaminofluorene has been limited to their appearance in rats, but we have observed many of the same types in several strains of mice subjected to similar treatment. The frequency of involvement of particular organs differed with species and with different mouse strains, but when they appeared in mice the lesions were like those of the rat, similarly located (12).

It has been mentioned above that acetaminofluorene may not itself be the active carcinogenic principle. Bielschowsky (2) has reported the appearance of tumors when aminofluorene is applied to the skin. Histological study of tissues from 6 rats and 7 mice developing lesions after aminofluorene feeding has revealed lesions that are essentially the same as those produced by acetaminofluorene (5).

Most of the distinct tumors observed have appeared in organs which were also the site of frequent irregular hyperplasia of cells of the same type as those producing the tumors. In each such organ it has been impossible to draw a sharp line of distinction between non-neoplastic hyperplasia and tumor formation. The hyperplasia never involved all parts of an organ or tissue uniformly, but appeared only in foci, which suggests that there was gradual development of some of these foci until large, well defined nodules were formed, or until sufficient penetration of adjacent tissues had taken place to classify the process as malignant growth. In portions of lesions which showed such "invasion," the cellular structure was sometimes indistinguishable from that of other lesions which were localized. No evidence was seen to suggest a sudden transformation of hyperplastic to neoplastic cells, or of benign to malignant cells. Many authors have expressed similar conclusions with respect to experimental skin tumors and Willis (9, 10) has collected evidence suggesting that human skin tumors do not arise by a single sudden transformation of epithelial cells.

The great frequency and invariable multiplicity of nodules in the liver may be the result of greater susceptibility of the liver cells to the effect of the carcinogenic agent or to more intimate contact of the agent with the liver cells than with those of other organs. Our observations do not clarify this problem. The findings of Bielschowsky (3) who reported that combined administration of allylthiourea and acetaminofluorene will produce benign and malignant tumors of the thyroid in rats, suggest that some factor in addition to the acetaminofluorene is important in the localization of the carcinogenic effect, and that this element may be provided or enhanced by certain types of stimulation of tissue growth. While it is possible that such factors may play a part in the development of all tumors following acetaminofluorene administration, it is impossible at present to identify them. The livers in our animals did not show frequent cirrhosis such as that found after feeding p-dimethylaminoazobenzene to rats, and we have seen no other morphological changes that might suggest a predisposing condition. Similarly, in other organs which were the site of proliferative changes, there was no reason to suspect any specific predisposing factor. Inflammation was associated with some of the pulmonary lesions, but a number were entirely without evidence of inflammation. Also, a number of inflammatory lesions in the lungs and elsewhere were not accompanied by any unusual proliferative changes.

The rarity of the development of tumors in any but epithelial cells following the administration of

acetaminofluorene, and the repeated localization of lesions in the same organs, suggest a specificity of the carcinogenic effect, possibly related to the metabolism or excretion of the substance. On the other hand, the appearance of nodules of two types of proliferating cells in frequent association in the same liver emphasizes the lack of complete specificity of the carcinogenic agent. The possibility that the agent acts merely by acceleration of a latent tumor-forming capacity of the treated animals, as has been visualized by Engelbreth-Holm (4) and others, cannot be excluded. Such a mechanism might account for the differences in frequency of the various tumor types in different strains of animals after administration of acetaminofluorene.

SUMMARY AND CONCLUSIONS

1. Oral administration to rats of small quantities of acetaminofluorene has been followed by the development of a wide variety of tumors in different tissues. Most of the tumors are derived from epithelial cells.

2. Most tissues that give rise to tumors are also the sites of nodular epithelial hyperplasia which is not distinctly neoplastic. No sharp distinction can be made between these hyperplastic nodules and the tumors formed by similar cells.

3. Malignancy can be recognized in some of the tumors by the occurrence of tumor cell infiltration and metastasis.

4. The factors determining the localization of the experimental tumors are not known.

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Distribution and Growth-Potency of Cells in a Transplantable Sarcoma*

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The biological and clinical characteristics of the Crocker mouse sarcoma 180 have in recent years been rather thoroughly studied, especially in relation to experimental procedures. It is common practice among most workers when making new implants to cut the "viable" tissue (usually considered to be the portion having a firm, pearly appearance) of the tumor, freshly removed from the mouse, into fragments about 2 mm. square. With the use of a trochar, one of these cubes is inserted subcutaneously into the axillary region of the host mouse. Within 7 days the implanted fragment grows to a mass of approximately 8 mm. in diameter, at which time it is considered suitable for experimental work or for dissection and re-implantation. If allowed to run its course, at the end of about 2 weeks the tumor attains a diameter of approximately 15 mm., and becomes ulcerous or encrusted. Usually, within 2 to 3 weeks after implantation, the host mouse dies, due presumably to necrosis toxemia; although complete regressions may occur. The weight growth curve of mouse sarcoma 180 so implanted is usually steepest between the fifth and eighth days, and the tumor is non-metastasizing.

In connection with certain experiments requiring a standardization of implanted tumor tissue, it became desirable to make a population and distribution study of the cells comprising this type of tumor at various stages of its growth-cycle, as well as of their growth-potency at such stages. The results of such a study are herein described.

PROCEDURE AND RESULTS

Technic.—When mouse sarcoma 180 is examined in fixed and stained histological preparation, two facts are immediately evident: (a) the cell population of the tumor is heterogeneous as to type, and (b) certain areas of the tumor differ from others in stroma characteristics and in cell distribution. Histological methods do not permit a ready assay of cell-types in experimental tissue at the time of its use, nor do they lend themselves to dependable cell-counting technics. Therefore, a system was sought which would permit sampling of experimental tumor tissue for cell density and cell distribution immediately before implantation. Throughout this study male mice of the inbred Rockland Swiss strain, 18 to 22 gm., were used as hosts.

Tumors were prepared by the trochar method described above. Suspensions were made by removing the whole tumor, of known age, from the mouse, and with the addition of 10 times its volume of Ringer's solution, thoroughly macerating it in a mortar without added abrasive. The resultant suspension was allowed to settle for about five minutes in a 15 ml. tapered test tube, during which time the gross fibrous material was carried down. The supernatant when drawn off in a syringe was found to be a suspension of free cells, which after thorough agitation were in relatively random distribution, and whose population density could therefore be calculated readily from cell counts of samples. It was found that this method of freeing the cells from the fibrous stroma gave a much larger yield of cells than procedures involving the passage of the macerated tissue through cheesecloth or surgical cotton.

An unstained suspension thus prepared, when examined in a hemocytometer under high-dry objective, was found to consist of two primary cell classes: (A) those which appear viable, having clearly defined cell walls, clearly defined nuclear walls, and a characteristic cytoplasmic translucency; and (B) those which appear necrotic, having ruptured or atypical cell walls, pyknotic or atypical nuclear walls, and a characteristically opaque cytoplasm.

The class of cells (A) which we have chosen to designate as "viable" was found further to consist of 5 types: (a) large cells, irregular in contour, often fibroblastoid and fusiform in appearance, with large spherical nuclei which may contain conspicuous nucleoli; (b) small cells, irregular in contour, with small spherical nuclei; (c) polymorphonuclear leucocytes; (d) non-granular cells resembling monocytes or lymphocytes; and (e) cells of groups (a) or (b) in some phase of mitosis.

The class of cells (B) which we have chosen to designate as "necrotic" cannot in our experience be subdivided as to type. Detritus which early in the study had tended to be confusing, ceased to be troublesome when the above-mentioned grinding and settling technic was employed. Particles in the suspension smaller in diameter than an erythrocyte were not counted as cells. Clumping or agglutination of cells also ceased to be troublesome when the above technic was used.

Shear (7), writing in 1936, stated that he was unable to distinguish living from dead cells in a tumor suspension. Later Belkin and Shear (1), on the basis of staining reaction to neutral red, distinguished three types of cells: (a) cells with diffusely stained cytoplasm; (b) cells with intensely stained nucleus; and (c) cells which remained unstained. They considered types (a) and (c) as viable, and type (b) as dead. Schrek (6) found that a dilute eosin solution added to the suspension aided in differentiating viable from necrotic cells.

We have found that, after some practice, these two arbitrarily defined classes of cells ("viable" and "necrotic") are distinguishable in the unstained condition clearly enough to permit reasonably accurate differential cell counts, and calculation of cell numbers per unit volume of fluid. Although differential counts in many instances were also made of all the cell subvarieties listed above, the data presented in this paper are based principally on counts of "viable" and "necrotic" cells. Results of the complete differential cell counts have not been included here, mainly because of the arbitrary distinction between "large" and "small" cells of the viable class. It was felt that until some functional significance could be attached to these cells which would establish them as distinct types, emphasis on the differential composition of the "viable" cells would be premature. It is of interest that the percentage of mitoses among the viable cells rarely exceeded 1 or 2 per cent during the entire life of the tumor. Since the observations were made on unstained preparations, it is possible that the earliest and latest stages of mitosis were not discernible.

With these methods for assaying the cell population in a given suspension, inocula may be set up whose absolute cell number is controllable and whose differential cell distribution is known approximately. Although a subjective factor enters into the making of count determinations, it has been found during the taking of hundreds of sample counts that estimates by two different observers on identical samples are the same to within plus-or-minus five per cent.

Graphs (A), (B), (C) and (D) in Fig. 1 give the data on the proportion of viable and necrotic cells in suspensions made from whole tumors of various ages, as well as from tumor tissue components of various ages. No attempt has been made to establish the quantitative statistical significance of the data.

POPULATION OF CELL TYPES AT VARIOUS STAGES OF TUMOR GROWTH

Suspensions were made of individual whole tumors at intervals in the age range of 2 to 14 days, and counts were made of viable and necrotic cells in samplings of such suspensions. The data so derived are plotted in Fig. 1 (A). It is seen that the young tumor is high in necrotic cells and low in viable cells. Between 6 and 8 days the proportion of viable cells has increased to a maximum, with a corresponding drop in necrotic cells. By 12 days this situation has again reversed itself, and the tumor at this time has the necrotic cell as its most populous type. Macroscopically the 12 or 14 day tumor gives signs of necrosis and ulceration.

The rather peculiar fact that the young tumor is high in necrotic cells warrants some discussion. The classical picture of the body's initial reaction to an implanted tumor fragment is essentially an inflammatory one. Within a few hours after implantation there is a marked aggregation of polymorphonuclear leucocytes in the connective tissue surrounding the fragment. This is succeeded by an infiltration of monocytes and lymphocytes, and then within 24 hours by a multitudinous influx of fibroblasts. The connective tissue surrounding the new implant immediately after inoculation becomes hyaline and subject to infiltration by the above-mentioned succession of wandering white cells, blood cells, fibroblasts, as well as of capillaries, and within a few days appears to become organized into a firm and expanding tissue which comes to constitute the main mass of the tumor. The original fragment does not change appreciably in absolute size or shape (although its cells start undergoing degenerative changes almost immediately after implantation) until about the ninth day when a fusion with surrounding necrotic tissues renders its outlines and structure somewhat amorphous.

The chemical and physiological relationship of the implant to the surrounding connective tissue is not known. Whether the cancer process consists of a multiplication of the cells of the introduced fragment, or whether the surrounding inflammatory connective tissue cells become cancerogenic, or whether two such processes act in combination, remains one of the prime enigmas of cancer biology.

It is quite evident, however, that for mouse sarcoma 180 there is no increase in mass of the originally implanted fragment, and that this fragment, indeed almost immediately after implantation, becomes cytologically degenerative. It is for the latter reason probably that the curve for necrotic cells in Fig. 1 (A) is very high for young whole tumors. At least two-thirds of the total mass of a 2-to-3-day-old tumor consists of the fragment originally implanted.

ripheral zone, usually considered to be the tumor's most viable tissue from the standpoint of transplantability, is pearly in appearance and of firm consistency (Fig. 5).

The relative proportion of each of these parts changes markedly throughout the growth history of this transplantable tumor. At 48 hours after implantation about two-thirds of the total tumor mass consists of white caseous core, with the intermediate zone absent or indistinguishable from the
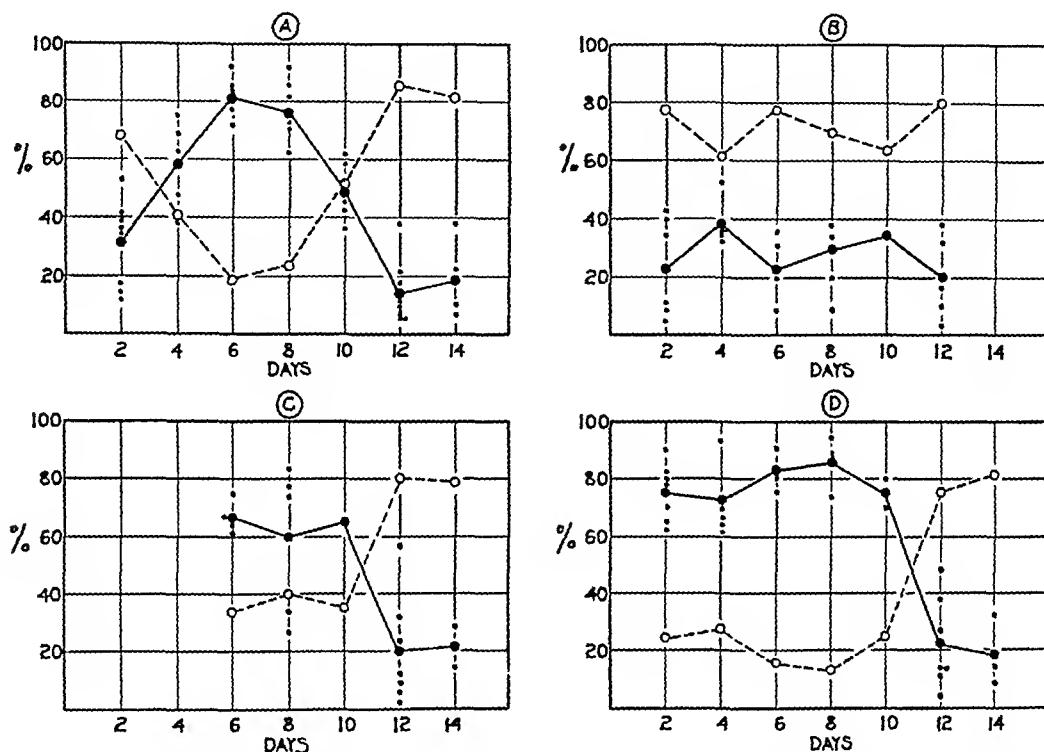
FIG. 1.—Proportion of viable and necrotic cells in suspensions made from tumors of various ages after implantation. Black points represent percentage of viable cells. White points represent percentage of necrotic cells. Each small point refers to a determination made on an individual tumor, and each was derived by counting 50 to 100 cells.

The corresponding individual determinations for necrotic cells may be derived as the difference between the viable cell level and 100 per cent. Large points represent the means.

(A)—describes cell proportions in suspensions made from whole tumors; (B)—from central core tissue; (C)—from intermediate zone tissue; (D)—from peripheral zone tissue.

DISTRIBUTION OF CELLS WITHIN THE TUMOR AT VARIOUS AGES

A 7-day-old mouse sarcoma, when bisected equatorially and examined macroscopically, is seen to consist of 3 distinguishable areas. The central core is white and caseous and lacking in vascularity, and constitutes, as we have said, the degenerative remains of the originally implanted fragment. An intermediate zone around the core is somewhat more granular, is vascularized, and its outer boundaries are not well defined. A pe-

vascularizing peripheral zone, which at this early stage is quite hyaline. At 7 or 8 days the central core has not changed appreciably in appearance or in volume, but massive intermediate and peripheral zones have developed. By 12 or 14 days the central core has lost its crisp delineation, as has also the inner boundary of the peripheral zone. An irregular granulation at this time characterizes both the intermediate and peripheral zones, and the tissue mass now appears to resemble that of the earlier intermediate zone, and has become

amorphous, with signs of necrosis and local hemorrhage becoming very evident.

An effort was made to tease out and separate tissues of these 3 zones from tumors of various ages; suspensions were immediately made, and differential cell counts taken. Fig. 1 (B), (C) and (D) present the data so obtained.

It is seen in Fig. 1 (B) that for the central core, the necrotic cell type is most numerous, and that its high level is maintained throughout the period from 2 to 12 days. Viable cells are proportionately low. Determinations at the 14-day level were not made because of the difficulty of dissecting out clearly defined core tissue at this stage.

Fig. 1 (C) shows data on suspensions from the intermediate zone. The intermediate zone does not become well enough defined for successful separation from the other zones until about the sixth day. Hence, data on this zone could be taken only from tumors 6-to-14 days of age. A general similarity between the cell population curves for the intermediate zone and those for the whole tumor (Fig. 1, A) is noted, both types of tissue being highest in viable cells at the 6-8 day stage, and highest in necrotic cells at the 12-14 day stage.

The curves in Fig. 1 (D), derived from peripheral zone suspensions, are also somewhat similar to those shown for the whole tumor (Fig. 1, A) and for the intermediate zone (Fig. 1, C), although there is at least one difference in the early portions of the curve. At 2 and 4 days the number of necrotic cells is significantly lower in the peripheral zone suspension than in the whole tumor suspension. This, on the other hand, is not unexpected in view of the above described central core involvement in the whole tumor data at these early stages. The peripheral tissue has no marked necrotic elements until after about the tenth day, when a general necrosis begins occurring throughout the whole tumor.

TUMOR INDUCTION CAPACITIES OF INOCULA OF KNOWN CELL NUMBER

It has been known since the advent of experimental tumor work that tumor cell suspensions may be employed for transplantation purposes. Earliest workers (8) knew that "small" and "large" inocula of tumor suspensions produced proportionately small and large tumors, after a specified interval of time. De Gaëtani and Blothner (2) found that the minimal inoculum necessary to induce grafts of the Ehrlich mouse carcinoma was in the order of 100,000 cells. Kahn and Furth (4) were able to transplant a 1,2-benzpyrene mouse tumor with as few as 50 cells. MacDowell (5) found that inocula of 300 leukemia cells would

produce takes in 10 per cent of the mice, and that inocula of 312,000 cells would produce takes in 100 per cent of the mice, while Furth and Kahn (3) have been able to transmit leukemia with a single cell.

The present experiment represents an effort to establish a standardizable relationship between the number of cells in the inoculum and the growth rate of the subsequent tumor mass. Early in the study it became evident that a number of procedural variables had to be tested for possible interference factors. For example, it was found advantageous to standardize the volume of each inoculum (with the exception of certain indicated instances) to 0.1 ml. This was necessary because it was observed that large variations in the absolute volumes of inocula would produce varying local concentrations of cells in the subcutaneous tissues. Thus, a large subcutaneous bleb (0.5 ml.) causes a greater cleavage in the connective tissues than does a small one (0.05 ml.); and, hence, as the liquid of the inoculation vesicle is resorbed, the plaque of cells so deposited in the connective tissue may vary in area and density. A million cells inoculated in a 0.5 ml. volume shows experimentally somewhat less growth than does an equal number of like cells suspended in a 0.05 ml. volume.

A standardization of the site of implantation was also necessary, presumably because of variations in the subcutaneous vascular anatomy or regional resistance factors in the body of the mouse. The procedure finally adopted was to inject the suspension through a No. 23 needle inserted under the epilated skin about ¼ of an inch below the posterior angle of the thoracic basket and about ¼ of an inch off the mid-line. The needle was projected thus subcutaneously to a point in the mid-rib region just under the conspicuous dermal vein in this area. That the inoculation was properly made in each case was easily confirmed by the visibility of this vein riding up over the surface of the resulting bleb.

In preparing suspensions of cells to the required concentration, a series of diluting media was tested. Among these were Tyrode's solution, blood plasma from the tumor donor mouse, supernatant fluid of a centrifuged tumor cell suspension, adult mouse heart juice, Ringer's solution, vitamin-fortified Ringer's solution, a 10 per cent solution of gelatin (7), and glycerin.

Standard Ringer's solution was finally chosen as the simplest diluting medium having no observable adverse or erratic effects on cells in suspension. The acceptability of Ringer's solution was further confirmed by allowing tumor cells so suspended to remain at room temperature for several

hours. Equivalent inocula (of equal volume as well as equal cell number) were removed from such suspensions at hourly intervals. It was found that such inocula did not decrease in potency for tumor induction up to about the third hour. Thereafter, the potency of the inocula began to diminish, due possibly to bacterial contamination or autolysis.

In order to test whether centrifugation affects the potency of the suspended cells, experiments were performed in which some inocula had been subjected to centrifugation and re-suspension, and others not. No growth interference was observed unless centrifugation was repeated 2 or 3 times, and unless the supernatant was wholly replaced by Ringer's solution. Excessive and repeated
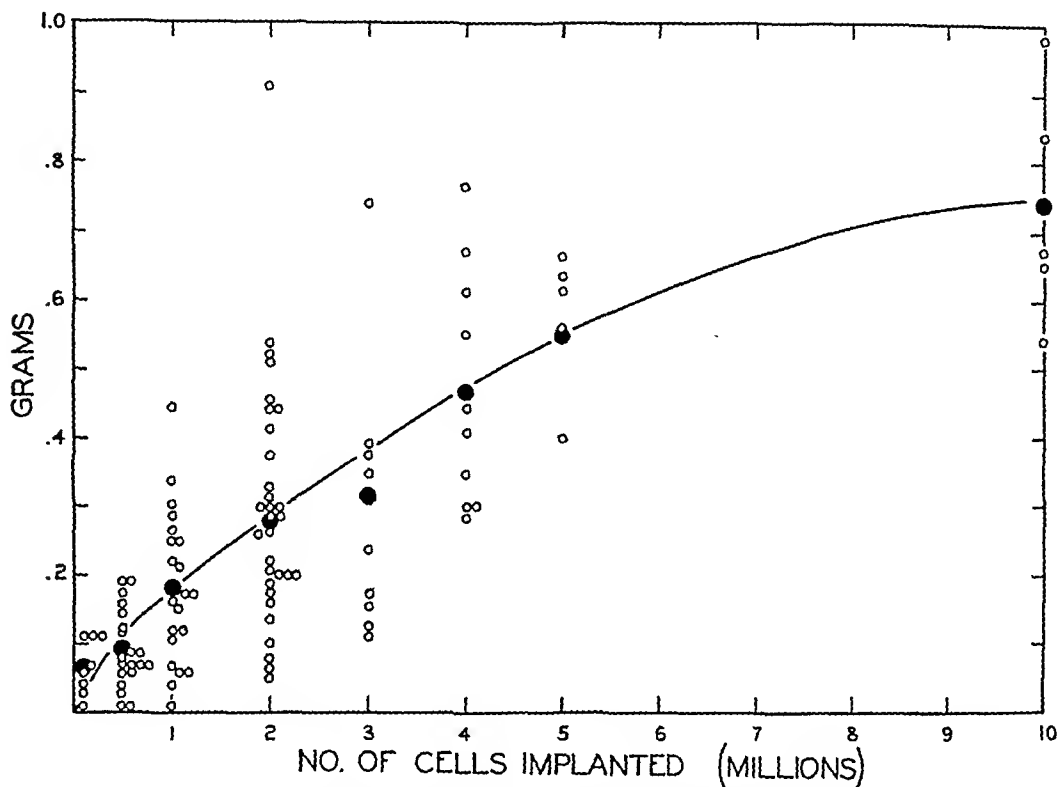
FIG. 2—Weight of tumor at the end of 7 days, plotted against the total number of cells in the inoculum. All inocula were taken from suspensions prepared from 7-day old tumors. Each white point represents one tumor. The black points represent the means.

Insofar as possible, asepsis was observed in dissecting out the tumors and in preparing the suspensions and dilutions; although asepsis in surgical procedures with mice is probably not as critical a desideratum as many workers have supposed. Suspensions known to have been accidentally contaminated during manipulation showed very little difference in resulting tumor growth from those known to be wholly sterile, so long as the time interval between contamination and inoculation was not excessive.

In preparing some of the very dense suspensions required for large-dose inoculations, centrifugation of the primary suspensions, followed by re-suspension in a fraction of the supernatant, was necessary.

packing does seem to reduce growth potency of tumor cells to some extent. Whether this is due to mechanical damage to cells, or to other factors involved in centrifugation, is not known. In any case, it was concluded that mild centrifugation and partial decantation of the supernatant is not harmful to the tumor cells and would not increase this general experimental error of the procedure.

The effect of grinding was also studied. Suspensions were made by macerating the tumor in a mortar in the usual way. Portions of such suspensions were subjected to continuous grinding for ten minutes. The growth potency of inocula of equal volume from the lightly and heavily ground preparations did not differ significantly, indicating

that the degree of cell damage, if such occurs in grinding, does not constitute an appreciable experimental hazard.

Employing the procedures suggested by the above discussion, repeated experiments were performed with inocula ranging in cell content from 1000 to 10,000,000. The suspensions were prepared solely from 7-day-old tumors of fragment origin.

curve is clearly evident. In the region of 500,000 to 5,000,000 cells the relationship between number of inoculated cells and resultant tumor growth is essentially rectilinear. The portion of the curve at 10,000,000 was not investigated in sufficient detail to warrant placing reliability on the exact form of the curve in this region.

Data for below 100,000 cells are not plotted in
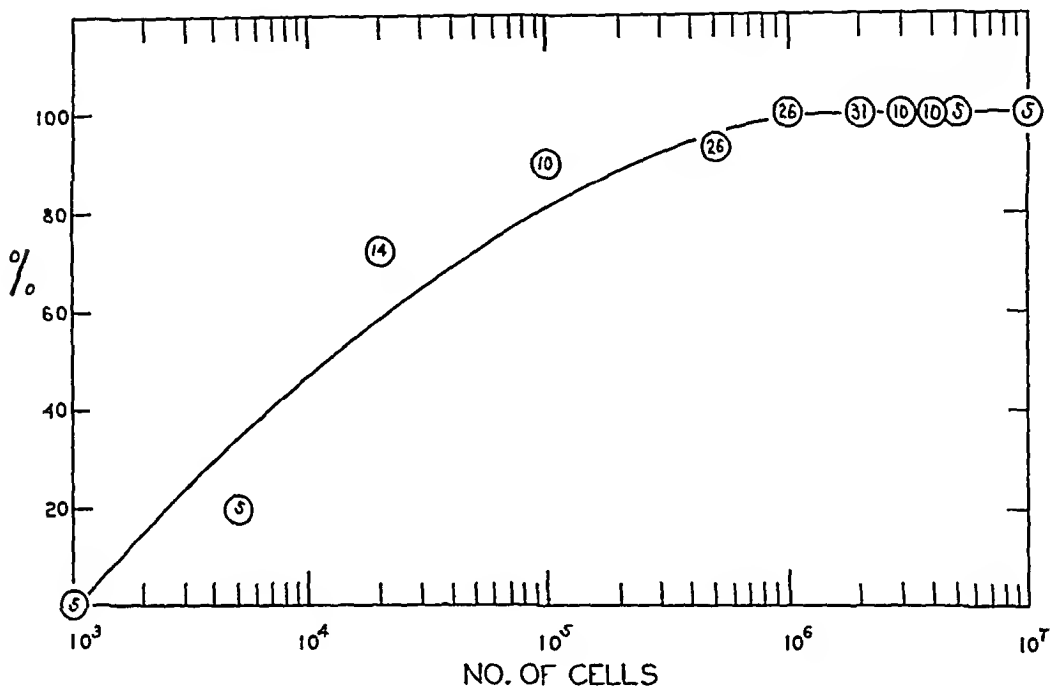
Fig. 3.—Percent of animals showing palpable tumor growths ("takes") at the end of 7 days, plotted against the total number of cells (from 7-day old tumors) in the inoculum. The abscissa range is logarithmic. The number of mice observed at each level is indicated within the circles.

The necessity for choosing donor tumors of standard age is evident from the cell distribution data presented in Fig. 1 (A). The volume of all inocula was 0.1 ml., with the exception of the experiments with inocula containing 10,000,000 cells. In this case, it was necessary to increase the volume of the inoculum to 0.2 ml. At the end of an arbitrarily chosen period of growth after inoculation (168 hours), the mice were killed, and the tumors were wet weighed. Tumor weight at the end of this growth period was taken as a measure of the growth potency of the inoculum. The number of non-takes was recorded.

The data so derived from the range of 100,000 to 10,000,000 cells are plotted in Fig. 2. Although the scatter of individual determinations is considerable at some levels, the trend of the mean

Fig. 2, due to the very low growth at the experimental end-point of 7 days, as well as to the increasing number of non-takes. Mice receiving small inocula (1,000, 5,000 and 20,000 cells) and not showing palpable tumors at the end of 7 days were not killed. A number of these developed tumors after several weeks, thus supporting the contention of Kahn and Furth (4) that some mouse tumors may be transmitted by smaller numbers of cells than was previously supposed. The number of takes above 500,000 was essentially 100 per cent. Below 500,000 the frequency of the takes begins to fall off, reaching zero between 5,000 and 1,000 cells. The percentage of takes as a function of the number of cells in the inoculum is plotted in Fig. 3.

TUMOR INDUCTION POTENCY OF CELL SUSPENSIONS MADE FROM TUMORS OF VARIOUS AGES

The data plotted in Fig. 4 are derived from a series of 4 experiments in which cell suspensions were made from whole tumors removed 2, 7 and 12 days after implantation of the fragment. Such suspensions were injected at a constant dose of 2,000,000 cells and at a constant volume of 0.1 ml.

sult in approximately equal amounts of tumor growth. For example, it is seen in Fig. 4 that suspensions made from tumors 7 days old were considerably more potent than those from the older or younger tumors. This is not surprising, in view of the data presented in Fig. 1 (A), since 2-day and 12-day tumors have the necrotic cell as their most populous type. It may be calculated from
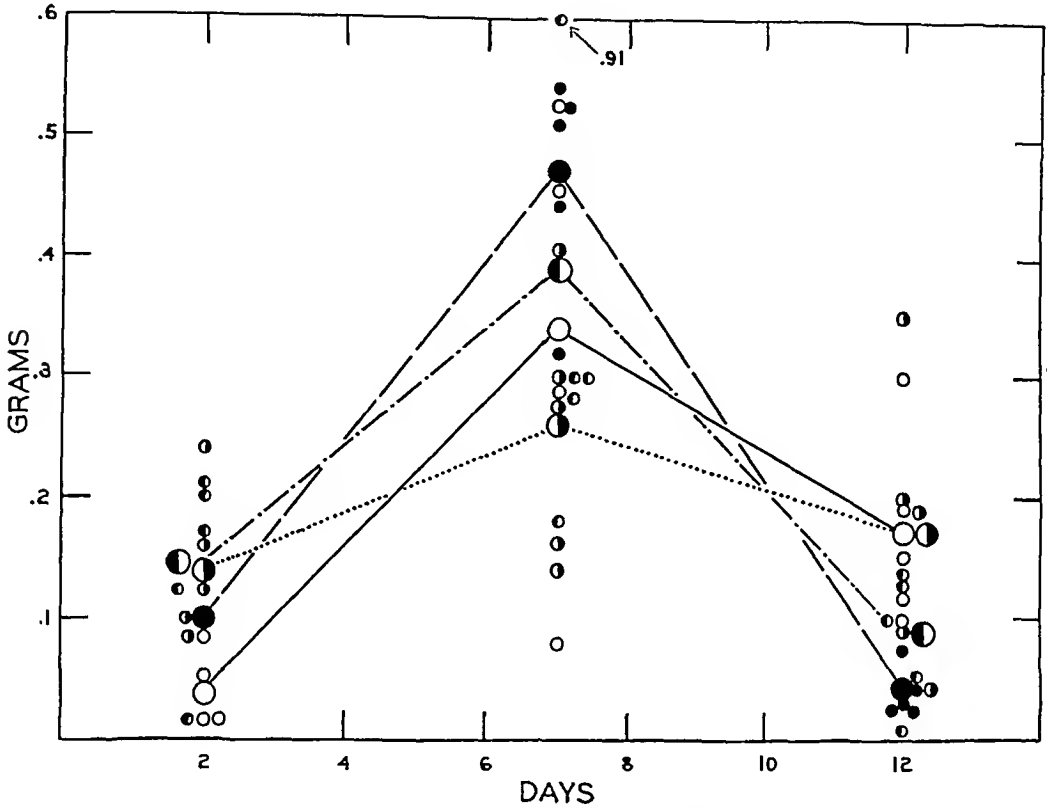
FIG. 4.—Weight of tumor at the end of 7 days after implantation, plotted against the age of the donor tumor from which the inoculation suspension was made. Small points represent individual tumor weights, large points represent the means. Four identically performed experiments are indicated with straight lines connecting the means. All inocula contained a fixed total number of cells (2,000,000).

In preparing these suspensions, a number of tumors of the appropriate age group were often macerated together in order to obtain the required number of cells. Because of this, only total cell counts were made on these suspensions. The differential cell data from Fig. 1 (A) were assumed to be applicable to such pooled-tumor suspensions.

A study of the data presented in Fig. 4 and in Fig. 1 (A) leads to the conclusion that equivalent doses of viable cells (that is, equal absolute numbers of viable cells), regardless of the percentage in the total suspension which they represent, re-

the data in Fig. 1 (A) that a 2,000,000 cell inoculum from a 2-day tumor would contain only about 600,000 viable cells, and one from a 12-day tumor would contain only about 300,000 viable cells. The growth from inocula of such numbers of viable cells corresponds approximately to the growth predictable from Fig. 2 for similarly sized doses of cells taken from 7-day tumor suspensions, known to consist preponderately of viable cells. At the 7-day level of tumor age, an inoculum of 2,000,000 cells would contain about 1,600,000 viable cells. Although the correspondence is not
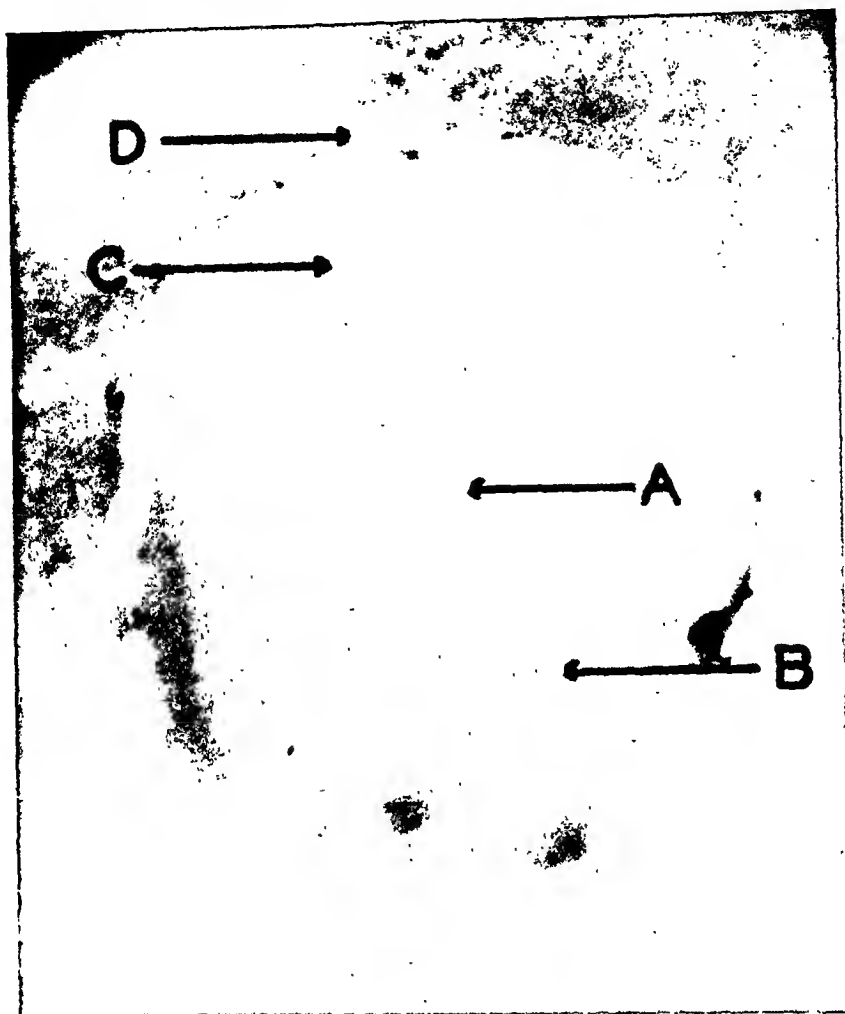
Fig. 5—Photograph of median section through 5-day old mouse sarcoma 180. (A)—central core; (B)—intermediate zone, just becoming evident at 5 days; (C)—peripheral zone; (D)—connective tissue. Mag. × 20.

perfect, it is seen in Fig. 2 that the weight data from such inocula are in the order of magnitude expected from 1,600,000 viable cells.

The correlation between growth potency and the number of what we have chosen to designate as viable cells, thus seems to support the validity of our distinction between "viable" and "necrotic" cells.

Growth potency of suspensions made from isolated tissue areas of the tumor is still under study.

DISCUSSION

It is evident that, with the use of inbred genetically pure mouse strains, the transplantation and growth of experimental tumors has become far more uniform than previously, but that even with such pure strains there is yet a high individual variation in response to tumor implantation.

A study of the distribution of the data in Fig. 2 shows that animals of the same strain, sex, weight, and age, fed on standardized diet, when inoculated with equal numbers of tumor cells of identical origin, and with the use of standardized injection technics, still show a relatively high variation in tumor growth response. This is also evident from the existence of a broad threshold in the region between 100 per cent takes and the beginning of the failure of all the mice to produce tumors. In the failure to observe greater uniformity of tumor

growth response, it seems necessary to assume that a considerable variation in tumor susceptibility exists even in homozygous animals.

The work described in this paper was undertaken as a prelude to a study of adjuvants and repressors of tumor growth. It appeared to us that if one could predict the normal growth behavior of a suspension of type-specified cells, experimentation both on the cells before inoculation and after inoculation could be better controlled than with the usual technic. The data presented in this paper reveal some variables which investigators have been prone to neglect. One variable, for example, which the experimenter must watch especially involves the distribution of cell types within the cancer tissue (either as suspension or fragment) which he implants for further work. On the other hand, even if this important factor is controlled, it is still evident that, due to individual variation among mice, large numbers of animals must be employed to establish the significance of any experimental procedure.

SUMMARY

Mouse tumor cell suspensions prepared by the use of a maceration technic have been studied. It is found that such freshly prepared and unstained suspensions contain a cell population of two distinguishable classes, which have been designated as *viable* and *necrotic*. The viable cells may be further subdivided as to type.

The proportion of viable and necrotic cells in suspensions made from whole tumors is found to vary regularly and significantly as a function of the age of the tumor, in the range of 2 to 14 days. Suspensions from very young tumors (removed 2 to 3 days after implantation) are high in necrotic cells and low in viable cells. The same condition applies to suspensions from old tumors (removed 10 to 14 days after implantation). In suspensions from tumors removed 5 to 8 days after implantation, the viable cells are by far the most numerous.

The central core of the tumor (*i.e.*, the implanted fragment) does not appear to change appreciably in size or shape from the time of implantation to about the tenth day. During the period between implantation and 10 to 12 days the cell distribution in the core tissue does not change significantly, a high and constant level of necrotic cells being found. The intermediate tissue zone is relatively high in viable cells during the 6-to-10 day period, high in necrotic cells thereafter. The peripheral tissue zone is high in viable cells from the second to the tenth day, thereafter becoming high in necrotic cells.

A technic is described for preparing cell suspensions of known cell number and cell type, which when implanted will give fairly predictable tumor growth rates.

The weight of the inoculated tumor after 7 days of growth varies as a function of the number of viable cells introduced. The smallest number of mouse sarcoma 180 cells producing a weighable tumor after 7 days of growth is of the order of 20,000, although inocula containing fewer cells may produce tumors if allowed to remain in the mouse for a longer period of time. The proportion of takes increases as a function of the number of cells introduced, within a specified range.

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Influence of 2,3-Dimercapto Propanol (BAL) on the Induction of Skin Tumors in Mice by 3,4-Benzpyrene

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Among the many agents influencing the rate of induction of skin tumors in mice with a standard carcinogen, hydrolyzing chlor-compounds (1), bromobenzene (2) and unsaturated dibasic acids (3) have been shown to either delay or prevent tumor formation. In attempting to explain why such diverse compounds have a common anti-carcinogenic effect, Crabtree (3) has suggested that their influence is mediated through a disturbance in sulfur metabolism. More specifically, he suggests that these three types of inhibitors react with sulfhydryl groups of the cell by the chemical process of condensation, oxidative coupling, or addition, thus making the sulfhydryl groups unavailable for normal cellular metabolism. Crabtree further postulates that the initial phase involved in the production of skin tumors with a standard carcinogen is the fixation of the carcinogen to the cell through combination with free sulfhydryl groups. If these free -SH groups have been previously blocked by an anti-carcinogen or if the anti-carcinogen competes with the carcinogen for cellular fixation, the carcinogen will be eliminated without producing its characteristic effects.

It was believed that additional evidence to support this theory could be obtained by another approach, namely, to supply the tissue with additional -SH groups which would compete with cellular -SH groups for reaction with the carcinogen. The compound 2,3-dimercapto propanol (BAL) is known to possess the property of competing with tissue -SH groups for chemicals forming stable -SH complexes (4). Therefore, the effect of BAL on the rate of induction of skin tumors in mice with 3,4-benzpyrene was studied.

EXPERIMENTAL

Albino mice, predominantly males, (National Institute of Health "Swiss") were used in these experiments. The mice were allowed water and Purina special laboratory chow at all times and were housed in an animal room maintained at a uniform temperature. All mice were treated as follows: Three days prior to the first treatment, the hair was clipped from the scapular region. Twice a week, on Monday and Thursday, a 0.3 per cent solution of 3,4-benzpyrene in ether containing 2 per cent liquid paraffin was applied to the interscapular region with a No. 3 brush. In certain of these mice 24 hours after the 3,4-benzpyrene treatment, that is, Tuesday and Friday of each week, 5 per cent BAL in an ointment base consisting of a mixture of "Carbowaxes" was applied
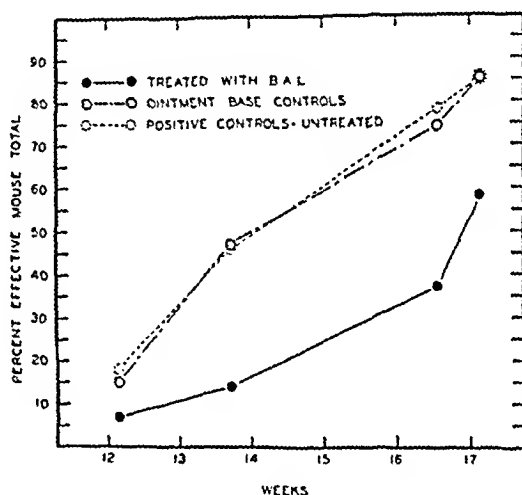
Fig. 1.—Incidence of tumors in mice skin painted with 3,4-benzpyrene.

over the scapular region. A quantity of 0.08 cc. of the BAL ointment measured from a 0.25 cc. syringe was used for each application.

In a preliminary experiment 70 mice were treated with the 3,4-benzpyrene of which 35 also received applications of 5 per cent BAL ointment. After 14 weeks of treatment, fewer mice receiving the 3,4-benzpyrene plus the BAL showed tumors than did those receiving the carcinogen alone. In order to check the apparently favorable results of BAL treatment obtained in this exploratory experiment and also to rule out any possible physical or chemical effects of the ointment base alone, the following experiment was started: Three com-

parable groups of 55 mice each were selected and were carried on experiment for 17 weeks. In addition to the 3,4-benzpyrene applications which all groups received, one of these groups was treated with BAL ointment as described above. The second group was treated with the ointment base without BAL, while the third group received no further treatment.

RESULTS AND CONCLUSIONS

The final results are shown in Table I. The progression of the experiment can be seen in Fig. 1.

TABLE I: INFLUENCE OF BAL ON INCIDENCE OF SKIN TUMORS AT 17 WEEKS IN MICE TREATED WITH 3,4-BENZPYRENE

Treatment	No. of mice at start	No. survived 17 weeks	No. with tumors	Percentage with tumors
BAL ointment, 5%	55	40	23	57.5*
Control, ointment base	55	34	28	82.4
Control, untreated	55	48	41	85.4

*Reduction in tumor incidence is statistically significant. ($p < 0.01$ by χ^2 test).

The results obtained clearly show the inhibitory influence of BAL on the induction of skin tumors in mouse skin treated with 3,4-benzpyrene. Whether the mechanism of this inhibition is explained by the removal of the carcinogen by -SH groups supplied by the BAL or whether the inhibition is due to a more nonspecific toxic effect of BAL can not be definitely stated. However, the observation of tumor inhibition by this agent deserves mention.

SUMMARY

The incidence of skin tumors in mice painted with 0.3 per cent 3,4-benzpyrene twice weekly for 17 weeks was materially reduced when these mice were treated with 5 per cent BAL ointment 24 hours after each painting with the carcinogen. Only 23 of 40 mice showed tumors after BAL treatment while 41 of 48 mice not treated with BAL showed tumors. Twenty-eight of 34 mice treated with the ointment base developed tumors, thus showing that the physical or physiological effects of constituents of the ointment base were not responsible for the reduced incidence of tumors after BAL.

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The Propagation of Filtrable Agents Producing Lymphoid Tumors And Osteopetrosis by Serial Passage in Chickens

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Although numerous experiments have shown that erythro- and myeloid leukosis may be transmitted by a filtrable agent (14), only a few reports have suggested that lymphomatosis may be transmitted by a similar agent.

By the use of filtrates of strain 2, Furth (9) produced what was thought to be a rare type of lymphomatosis. It was characterized by the appearance, in the blood and many organs, of large lymphocytes with occasional formation of lymphoid tumor-like nodules, endothelioma and severe anemia. Pentimalli (16) described a readily transplantable lymphoid tumor strain. Based upon results obtained from filtration, desiccation, and glycerination experiments, he concluded that the strain did not contain a filtrable agent. Olson (15) developed a tumor strain having similar characteristics; however, he did not report on attempts to transmit it by cell-free material. The propagation of 4 lymphoid tumor strains by cell transplantation from cases of naturally occurring visceral lymphomatosis was reported by Burmester and Prickett (3). These strains were similar among themselves and to those of Pentimalli (16) and Olson (15) in that the type of involvement was similar, the incidence of tumor takes was high and the rate of tumor growth was rapid. Brewer and Brownstein (2) have also reported on the rapid transmission of visceral lymphomatosis with suspensions of tumor pulp.

Burmester, Prickett, and Belding (4) in a series of 3 experiments demonstrated the presence of a filtrable agent in the lymphoid tumor originally studied by Olson (15) and obtained at this Laboratory in June, 1942 and since designated here as RPL 12. This filtrable agent failed to induce tumors at the site of inoculation in a short period of time (inocula-containing cells produce tumors at the site of inoculation) but produced within 6 months' time a high incidence of osteopetrosis and lymphomatous tumors of the viscera.

The object of this paper is to report on the propagation of the filtrable agents of the lymphoid tumor strain RPL 12 during 6 serial passages in young chickens and to describe the gross manifestations obtained in the various passages, with different routes of inoculation, and with different types of donors and preparations.

MATERIALS AND METHODS

In the work to be reported here two different types of inoculum were used. The serial passages were made with tumor material or plasma rendered cell-free by centrifugation and filtration. At the same time supplementary inoculations were made in certain instances with tumor cell suspensions or whole blood of the same source as the cell-free preparations.

Preparation of filtrates.—The cell-free extracts of lymphomatous liver tissue were prepared by homogenizing the tumor in 7 parts 0.85 per cent NaCl solution in a Waring Blendor for 20 minutes. Examination of samples by direct and dark-field illumination of wet specimens and fixed smears processed with Wright's stain revealed that after 8 minutes of homogenizing very few intact cells were found, these being mature erythrocytes, but a large number of free nuclei were seen. After 16 minutes no intact cells were found, the number of free nuclei was much less and the innumerable particles seen in the suspension were much smaller than in samples taken after 8 minutes.

For the two inoculations of the second passage Seitz clarifying filters, K1 and K7, were used. The former was more retentive than the latter. Microscopic examination of these filtrates failed to reveal intact cells or free nuclei. Inoculum for the third and fourth passages was prepared by centrifuging the homogenate for 20 minutes at about 3,000 RPM and then filtering through a Seitz S1 pad. The sixth passage was made with material prepared in a similar manner except that 7 parts of

Tyrode's solution at pH 7.1 was used and the centrifuged supernatant was filtered first through a preliminary and then through a regular Mandler candle.

The plasma was obtained from blood which had been withdrawn from the heart of donors into a syringe containing 0.1 volume of heparin solution having a concentration of 0.4 gm. per 100 ml., in 0.85 per cent NaCl solution. It was separated from the cells by centrifugation and then filtered through a sterilizing Seitz S1 pad for passages 1, 3, and 4. A Jenkins-Fisher sterilizing filter was used for passage 5 inoculum.

Forty-eight hour broth cultures of *Serratia marcescens* were used to test all Seitz S1 filters after their use with the extracts and the regular Mandler candle before and after it was used for liver extract. In all cases 1.0 ml. of the filtered broth cultures failed to seed tryptone broth; whereas the unfiltered portion produced typical growth in all tests.

For the extra inoculation of passage 3 the plasma was diluted with equal parts of sterile distilled water. Part of it was then subjected to high-speed centrifugation. It was spun in an angle centrifuge for 20 minutes at 5,000 RPM and the supernatant transferred to six 14-ml. lusteroid tubes and spun at 19,000 RPM (27,000 times the force of gravity). After centrifuging for 2½ hours the contents of each tube was divided into halves, the upper portions were combined and transferred to other lusteroid tubes and the lower portions were similarly combined. The two fractions were again spun for 2½ hours at 19,000 RPM, after which the upper half of the contents of each tube, containing the first upper fraction, was carefully transferred to a serum bottle for inoculation and the lower portion, containing the first lower fraction, was transferred to a second bottle. Gelatinous pellets or sediments were not obtained in either of the two high speed centrifugations.

Preparation of cellular inoculum.—The tumor cells suspensions were prepared by a method already described (3). The whole blood as used for inoculation was not treated after its collection from the donor.

Experimental birds.—The recipient chicks were pedigreed and obtained from matings of an inbred line (line 15) of chickens relatively susceptible to lymphomatosis yet which developed only a few or no cases when maintained under quarantine (17).

The chicks were inoculated at 1 to 3 days of age with 0.25 to 0.5 ml. of the cellular inoculum or 0.5 to 1.0 ml. of the cell-free preparations by the intraperitoneal route except where otherwise indicated. The different inoculation groups of the same passage were reared in the same battery and pens; however, birds of different passages were maintained in separate quarantine pens.

Two groups of non-inoculated control chicks, from the same matings used for the inoculations, were maintained for the first 90 days after hatching in a similar but separate quarantine pen from the inoculated chicks. During the second period of about 90 days, one control group was maintained with birds of the first passage and a second group was kept in the same pen with birds of the extra inoculation of passage 3.

All experimental and control birds were examined after they had died, or were killed to serve as donors, or at the termination of the experiment. All except 2 groups were terminated at 6 months of age. Because of a misunderstanding the birds of the fourth passage were killed at 5 months of age and those of the fifth passage were terminated at 3 months of age. All diagnoses were based on gross alterations observed at necropsy and at periodic clinical examinations. In addition, tissues of all donors were examined microscopically.

PASSAGES AND RESULTS

A summary of the transmission data for the various inoculations and passages is presented in Table I. The results of inoculations representing the first passage of this series have already been presented elsewhere (4). They are included here to facilitate comparison with data of subsequent passages. The inoculum used to initiate this series was plasma obtained from 2 chickens which had received an implant of Strain RPL 12 tumor cells 7 days previously. These donors had large intramuscular tumors at the site of inoculation and diffuse involvement of several visceral organs. The filtered plasma produced tumors in 86 per cent of those inoculated by the intravenous and by the intraperitoneal routes. Most of the birds had tumors in the viscera but many also had osteopetrosis.

Two cases from the first passage were used as donors at 190 and 192 days of age. Both had lymphomatous tumors of the viscera but only one showed evidence of osteopetrosis. Filtrates from both donors produced a high incidence of tumors. The Seitz K7 filtrate of the donor having only visceral tumors produced osteopetrosis in 37 per cent of the recipients, while the Seitz K7 filtrate of the donor with osteopetrosis produced this tumor in 53 per cent of those inoculated. However, osteopetrosis did not occur in a group that had received the Seitz K1 filtrate prepared from the latter donor.

For the third serial passage again 2 donors were

used. Both arose in the group of the second passage that had been inoculated with liver tumor filtrate from a bird showing only visceral tumors. The first donor for the third passage had both osteopetrosis and visceral tumors, and was 148 days of age when sacrificed. Filtered plasma and liver homogenates reproduced a high incidence of both pathologic alterations in the recipients. The

filtered liver extract developed lymphoid tumors in the viscera; whereas, only 38 per cent of the plasma-inoculated group developed similar tumors.

The fifth passage was made with plasma of a chicken of the previous passage that had been inoculated with plasma and had developed severe lesions of osteopetrosis but showed no evidence of visceral involvement. The recipients were main-

TABLE I: TRANSMISSION DATA FOR THE SERIAL PASSAGE OF A LYMPHOID TUMOR WITH FILTRATES AND COMPARATIVE INOCULATIONS WITH CELL SUSPENSIONS

Passage No.	Donor used			Inoculum		No. chicks inoc.	% with tumors			Total % pos.	Average survival visc. cases (days)
	Days after inoc.	Diagnosis			Filter used		Bone	Visc.	Nerve		
		gross	micro.	Source							
1	7	P. V†	P, V, 0	Whole blood	None	4*	0	100	0	100	7
		P, V	P, V	Whole blood	None	4	0	100	0	100	13
				Plasma	Seitz S1	14*	43	57	7	86	132
				Plasma	Seitz S1	14	43	71	0	86	165
				Not inoculated—Contro's		14	0	0	0	0	...
2	189	V	V	Liver tumor cells	None	14	7	64	7	72	71
				Liver homogenate	Seitz K7	19	37	68	5	79	147
2 (extra)	187	V, 0	V, 0	Liver tumor cells	None	15	20	87	0	87	84
				Liver homogenate	Seitz K7	15	53	87	7	87	143
				Liver homogenate	Seitz K1	15	0	60	7	60	137
3	145	V, 0	V, 0	Whole blood	None	8	0	63	0	63	43
				Plasma	Seitz S1	20	50	65	5	85	131
				Liver tumor cells	None	10	30	80	0	80	75
				Liver homogenate	Seitz S1	18	33	67	17	78	128
				Not inoculated—Controls		17	0	0	0	0	...
3 (extra)	159	0	0	Whole blood	None	13	15	69	0	77	119
				Plasma	Seitz S1	16	44	69	6	69	135
				Plasma Centrif.	Upper fract.	20	50	70	0	85	136
				Plasma Centrif.	Lower fract.	20	45	65	0	80	131
4‡	43	V, 0	V, 0	Plasma	Seitz S1	16	19	38	12	63	103
				Liver homogenate	Seitz S1	17	12	77	0	77	104
5‡	57	0	0	Plasma	Jenkins	14*	7	29	0	36	...
				Plasma	Jenkins	14	0	7	0	7	...
6	96	V	V	Liver homogenate	Mandler	18	83	95	0	95	140
Total and average percentages for cell-free preparations, exclusive of passages 4 and 5						189	44.4	70.4	4.8	81.0	137.0
				Not inoculated—Controls		31	0	0	0	0	...

*Inoculated by intravenous route, all others intraperitoneal route.
†P = Intramuscular tumors. V = Visceral tumors, O = Osteopetrosis.
‡Passages 4 and 5 were terminated at 5 and 3 months, respectively, all others were terminated at 6 months of age.

second donor showed evidence of only osteopetrosis at 162 days of age. Since the liver was not tumorous only filtered plasma was used. This inoculum produced a high incidence of visceral tumors and osteopetrosis. A difference in the incidence of the two manifestations between the upper and lower high-speed centrifuged fractions of plasma was not obtained.

The fourth serial passage was made with plasma and lymphomatous liver of a 44 day old bird. This donor had been inoculated with filtered plasma of the 148 day old donor of the third passage. By 5 months of age both groups of this passage developed a high incidence of tumors, although there was a marked difference between the two groups in the percentage with visceral tumors. Seventy-seven per cent of the chickens inoculated with

tained for an experimental period of only 96 days, during which time 3 of 28 birds inoculated had died with tumors and 3 others were found to have tumors when they were killed on the date of termination.

Birds of the sixth serial passage received a Mandler filtrate prepared from the liver of one of the three birds having tumors at termination of the fifth passage. Eighty-three per cent of the chickens that received this inoculum developed osteopetrosis and 95 per cent (all but one) had tumors in the viscera.

The total tumor incidence for all groups that were inoculated with filtrate and were maintained for 6 months was 81.0 per cent. Most of these (88 per cent) had tumors of the viscera and they died on an average of 137 days after inoculation. About

one-half (55 per cent) had osteopetrosis and only a few (6 per cent) had neurolymphomatosis.

None of the chickens of the two non-inoculated control groups showed any evidence of tumors or other manifestation of lymphomatosis.

The pathological manifestations obtained with filtrate inoculations of the 6 passages were similar to the visceral tumors and osteopetrosis previously described as the result of inoculations with cell-free preparations of this tumor strain (4). Massive lymphomatous tumors of the viscera occurred in all groups, and osteopetrosis occurred in all except 2 groups inoculated with filtered material. One to 3 cases typical of neurolymphomatosis were observed in 8 of the total of 15 groups inoculated with cell-free material.

Almost half of the positive cases had more than one type of involvement. Of the 153 cases obtained in the filtrate-inoculated birds held for 6 months, 67 had a combination of osteopetrosis and visceral tumors, 66 had visceral tumors without osteopetrosis and only 17 had osteopetrosis without gross evidence of visceral tumors. Of the 9 cases which had nerve involvement all but 3 also had osteopetrosis or visceral tumors.

TABLE II: GROSS INVOLVEMENT OF VISCERAL ORGANS AFTER INOCULATION WITH CELL-FREE MATERIAL

Passage No.	No of cases	Percentage distribution of Lesions among organs of lymphomatous birds						
		Liver	Spleen	Kidney	Gonad	Heart	Prov.	Perit.
1	11	100	82	73	18	9	9	9
2	28	97	82	53	25	7	4	0
3	51	100	73	39	24	4	4	0
4	15	100	80	80	27	20	0	0
5	5	100	80	80	20	0	0	0
6	17	100	88	76	12	18	0	6
Total	127	99	79	57	22	9	3	2

Gross tumor involvement of the various visceral organs in chickens that died after inoculation with cell-free material is summarized in Table II. Cases showing any visceral involvement almost invariably had lymphomatous livers. Most of them also had spleen and kidney involvement, followed by tumors of the gonad, heart, proventriculus and peritoneum in frequency. Other organs were occasionally involved. No apparent difference in the frequency of involvement of any organ was noted between the various passages, between different routes of inoculation, types of donors used or preparation of inoculum.

Study of tissues from all the donors and a limited number of recipients showed that the microscopic alterations found were uniformly typical of visceral lymphomatosis (8, 11) and osteopetrosis (1, 4, 10).

DISCUSSION

It is apparent that no significant trend or change occurred in the manifestations of this agent during the 6 passages. The type of lesions remained the same, and the incidence of osteopetrosis, visceral tumors, and neural involvement remained at about the same level, although there was a suggestion of an increase in activity since the lowest incidence of osteopetrosis and of visceral tumors occurred in the first and second passages, while the highest incidence occurred in the last or sixth passage. The average age at death was also remarkably similar for the 11 filtrate inoculated groups maintained 6 months.

Data presented in Table I indicate that the rate of tumor growth from transplants of tumors induced by filtrates was much slower than that from transplants of tumors that had been propagated in series with tumor cells, i.e., in serial passage with cellular inoculum. In inoculations of the first 3 passages, groups of chicks were also injected with whole blood or tumor cell suspensions from the same source as the filtrate preparations. Whole blood of birds with 7 day intramuscular transplants produced visceral tumors and death of all birds in an average of 7 (intravenous route) to 13 (intraperitoneal route) days' time with no evidence of osteopetrosis (Table I, passage 1). In contrast, chicks injected with whole blood from lymphomatous and osteopetrotic cases produced by filtrates of the second passage, developed an incidence of 63 and 69 per cent visceral tumors, respectively, and the age at death was prolonged to an average of 43 and 119 days, respectively. Two cases of osteopetrosis occurred in the latter group. Cellular suspensions of lymphomatous livers were also used in inoculation of the second and third passages. The incidence of visceral tumors was high and cases of osteopetrosis appeared in the 3 inoculated groups. The birds died on an average of 84, 71, and 75 days after inoculation.

When birds were inoculated with cell suspensions, prepared from tumors that had been induced by a cellular inoculum, tumors were produced in all birds, the average survival was 7 and 13 days for 2 routes of inoculation (passage 1) and osteopetrosis was not evident;[1] whereas birds in-

[1] This is typical of results obtained at this Laboratory during 55 serial passages of this tumor strain with cellular preparations. All of the 548 birds used in these passages developed tumors, the average survival time was 9.52 days and gross evidence of osteopetrosis was not observed. Microscopic alterations indicative of osteopetrosis were observed in one case (a donor for passage 1 herein described). The lack of appearance of gross bone involvement may have been due to the short survival period, which did not allow sufficient time for grossly visible bone alterations.

oculated with cell suspensions prepared from tumors that had been induced by a cell-free inoculum developed similar tumors, but the incidence was lower, the average survival time was much longer (43 to 119 days) and osteopetrosis was present in all but one group. This longer survival time, which is directly related to the rate of tumor growth and malignancy may be related to the fact that the filtrate-induced tumors used in the transplants took much longer to develop (average of 137 days) than did tumors grown in serial passage with cell transplants (average of 10 days).

A similar difference in results between cell-free preparations of tumors induced by cellular inoculum and cell-free preparations of tumors induced by filtrates was not obtained in these passages. Actually, the filtrate used for the sixth passage produced the highest incidence of tumors and the third passage filtrate group had the lowest age at death (excluding passage 4). However, the differences between these values are small and insignificant. Since the apparent activity of the filtrates remained at about the same level, whereas the apparent malignancy of the tumor cells was much greater in the first than in subsequent passages, one may infer that a positive relation between the malignancy of the tumor cells and virulence of the tumor agent was not obtained in the present experiment.

Tumors in birds that received filtrates were presumably due to a filtrable agent or agents contained therein; however, tumors in birds that were injected with cell suspensions may have been due to direct multiplication of the transplanted cells, or due to an agent within the cells injected or a combination of both. It is significant that except for the first passage (from transplanted tumor) the cellular inocula were no more effective in producing lymphomatous tumors and osteopetrosis than filtrates prepared from the same source.

Although cases of neurolymphomatosis occurred only in inoculated groups the incidence is low and of doubtful significance. Its occurrence may or may not be due to factors other than the inoculum.

During the course of the serial passage inoculations a limited number of transmission variables were tested in a preliminary manner. In the first and fifth passages the intravenous route of inoculation was compared with the intraperitoneal route. The differences obtained were small and not consistent. In the first test the intravenous route caused death in a shorter time but the intraperitoneal route produced the higher incidence of visceral tumors. In the second test a higher incidence in a 3 months' period was obtained with the intravenous route. No difference was obtained in either test in the occurrence of osteopetrosis.

In passages 3 and 4 filtered plasma was compared with the liver filtrate from the same donor. No differences were obtained in the passage 3 test with respect to incidence of osteopetrosis, visceral tumors, or to length of survival time. In passage 4, which was terminated at 5 months, the incidence of visceral tumors in the liver filtrate group was almost twice that in the plasma-inoculated group; however, there was but little difference in the total incidence of tumors. It is thus apparent that an active agent was present in both the lymphomatous liver and in the blood plasma. Differences in the concentration of the active agent could not be estimated because the experimental design does not lend itself to such analyses.

An attempt was made to concentrate the agent in plasma by two centrifugal runs at 19,000 RPM (27,000 times gravity). No difference in transmission was obtained between the upper and lower one-half of the contents of the centrifuge tubes. This result was to be expected since no pellet or other evidence of separation was obtained during this centrifugation. In later experiments, working with muscle tumors, Burmester (5) obtained evidence of sedimentation of the same agent or agents by centrifugation at 19,000 RPM and at 40,000 RPM.

Homogenized lymphomatous liver tissue filtered through a Seitz sterilizing filter in the third passage produced as many tumors as similar material filtered through Seitz clarifying K1 and K7 filters for inoculation in the second passage. Although comparisons of these filters must be made with results obtained with different donors, it would appear that under the conditions of these inoculations the fine filters did not remove much more agent than the coarser ones.

There were variations in the pathological alterations of the donor which were not correlated with similar variation in the recipients. The donors used for passages 2 and 6 had massive lymphomatous involvement of the viscera but no gross or microscopic evidence of osteopetrosis; however, 37 per cent of the birds of passage 2 and 83 per cent of those in passage 6 developed osteopetrosis. This incidence was as high as, or higher than, other passages in which the donor had osteopetrosis.

Donors used for the extra passage 3 and for passage 5 had osteopetrosis without gross or microscopic evidence of lymphomatous involvement of the viscera, yet a high percentage of the recipients developed visceral tumors. Although no attempt was made to separate the two manifesta-

tions during several serial passages, there was no indication of a tendency for one manifestation or the other to become predominant when a donor with only one type was used.

Two explanations may be presented: (a) the two manifestations are due to one and the same agent, and tissue resistance or other similar factors determine the type of involvement obtained, or (b) two separate agents are responsible for the two different manifestations and the alterations obtained are due to the relative activity of each agent. It has already been suggested that osteopetrosis and lymphomatosis may be due to different agents (4). Further evidence of a separate etiology was obtained by differential centrifugation studies (5). The "masked" or "latent" nature of the agent of osteopetrosis was noted by several investigators (1, 7, 10). A similar phenomenon has been demonstrated for Rous tumor virus (13), the Shope papilloma virus (12), and has been suggested for other agents of the avian leukosis complex (7). Thus, there is some evidence suggesting that osteopetrosis and lymphomas of the viscera are produced by different agents, and that either may remain latent in recipients and become overt in subsequent passages.

The results of 8 different inoculations and 2 control groups furnish conclusive evidence that an agent or agents passing through bacteria-retaining filters will induce the formation of osteopetrosis and lymphoid tumors of the viscera. The incidence of grossly visible tumors was high (69 to 95 per cent) in all groups inoculated with the filtrates and maintained for 6 months; whereas no evidence of tumors appeared in two control groups.

Sterilizing Seitz S1 filters were used for the preparation of 7 filtered inocula tested. All filters when tested after filtration of the inocula were found to retain *Serratia marcescens* completely. An 8-pound Mandler candle filter was used for another inoculation. This filter was tested before filtration of the inoculum and again after it was cleaned and resterilized. In all cases, filtrates from 48 hour broth cultures of *Serratia marcescens* were sterile. A Jenkins-Fisher filter[2] was used for the ninth filtrate. This particular filter was not tested for its retention of bacteria; however, 6 filters of the same type and chosen at random were found to completely retain *Serratia marcescens*.

Since all passages after the first were made with filtrates from donors that had received only filtered material, it may be assumed that the agent was propagated in the host as a result of the action of the agent or agents.

[2]Obtained from Fisher Scientific Company, Pittsburgh, Pa.

In working with visceral tumors from cases of naturally occurring lymphomatosis, Burmester and Denington (6) were able to produce a high incidence of lymphomatous tumors with cell-free preparations from 5 of 10 of the original tumors. One of these preparations also produced osteopetrosis. Four tumors were propagated in serial passage with cellular preparations (7). Filtrates prepared from three propagated tumors produced a high incidence of lymphoid tumors within a period of 200 days. The transmission and pathological characteristics of these three strains (RPL 18, 20, and 21) appear to be similar (except for a variation in the incidence of osteopetrosis) to the tumor strain used for experiments reported herein (RPL 12).

SUMMARY AND CONCLUSIONS

1. The filtrable agent or agents inducing osteopetrosis and lymphoid tumors of the viscera were propagated through 6 serial passages in chickens.

2. The incidence of tumors and average survival time were quite uniform for the several filtrate inoculations and passages. An average of 81 per cent of all birds inoculated showed some gross involvement and they died on the average in 137 days. Of the total positive cases 55 per cent had osteopetrosis, 87 per cent had visceral tumors, and 6 per cent had neurolymphomatosis.

3. Results obtained from inoculation of chicks with tumor cell suspensions and filtrates prepared from the same tissue suggest that there was no relation between the malignancy of the tumor cells and the virulence of the agent.

4. After the first passage, filtrates were as effective as cell suspension in producing visceral tumors, and the filtrates invariably produced a higher incidence of osteopetrosis.

5. Filtrates appeared to be about as effective by the intraperitoneal route as by the intravenous route.

6. Filtered plasma of tumor-bearing birds produced about as high an incidence of tumors in recipients as did filtrates of lymphomatous livers.

7. Neurolymphomatosis appeared in 8 of 15 groups inoculated with filtrates but the incidence was not significant.

8. Donors showing only osteopetrosis produced about the same incidence of visceral tumors and osteopetrosis in recipients as donors with only lymphomatous visceral tumors or those with a combination of the two manifestations.

9. Conclusive evidence is presented that this lymphoid tumor contains a propagative agent or agents that will pass through bacteria-retaining

filters and will induce a high incidence of osteopetrosis and visceral tumors in chickens within 6 months' time. The latter tumors have thus far been indistinguishable from the tumors seen in cases of naturally occurring visceral lymphomatosis.

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The Mast Cell Reaction of Mouse Skin To Some Organic Chemicals*

I. Estimation of the Relative Number of Mast Cells in Normal Mouse Skin

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Quantitative determination of the etheral sulphuric acids in the granules of the mast cells in small pieces of tissue would be of considerable value, but unfortunately no micromethods for such extractions and analyses have yet been devised. As matters stand, morphologists have thus to perform a rough estimation of the number of mast cells and their granule content from microscopic slides. The counting of mast cells per tissue unit presents no difficulties *per se*, but the estimation of the amount of metachromatic granules is rather subjective. However, when large differences exist in the number of mast cells and the granule content, such rough methods seem to give conclusive results (6, 7), but minor differences are apt to be overlooked. The methods outlined above are essential for studying the mast cell reaction to different agents. Without entering more closely into the rough quantitative data supplied by earlier authors (1, 2), a more detailed study will be presented in this paper, including a fairly reliable counting technic suitable for experimental work.

Whenever quantitative methods are applied for the demonstration of biological reactions, many questions will arise as to the validity of the control material. For instance, is it possible to determine a "normal" number of mast cells per cu.mm. in the skin of mice? How are they related to age, sex, body weight, nutritional conditions, and so forth? How large are the individual variations in litter mates? *Are the individual variations small enough to permit a reliable "normal value"* in the same region in the skin of mice, which in other respects are comparable?

Furthermore, we recall that some mast cells are rich in granules and will be heavily stained, whereas others are poor in granules and consequently are more difficult to discern. According to Hellström and Holmgren (3) it is desirable to perform the counting in thick sections (about 100 μ)

in order to reduce the sources of error, but in such sections granule-poor mast cells are easily overlooked. Thus we are compelled to count these cells in sections of different thickness and the technic must be varied with regard to the material and scope of investigation. Many mast cells in the skin contain only a few small granules, and therefore comparatively thin sections have to be used.

Most skin lesions chemically induced involve inconvenient secondary changes such as edema and inflammatory cell reactions, resulting in an increase in volume of the dermis. A mechanical counting of the number of mast cells per cu. mm. of dermal connective tissue could easily lead to an erroneous decrease in the number of such cells. To avoid the error due to such secondary volume increments, we have correlated the total number of dermal mast cells with the measured length of the epidermis. This is apparently the method of choice under such circumstances.

The methods here reported are specially devised for experimental purposes, *viz.* for further studies on the effect of carcinogenic hydrocarbons, and will deal only with the number of mast cells in the skin of the interscapular region.

TECHNICAL DETAILS

A stock of common Swiss albino mice was registered as usual with regard to age, weight and general condition and fed the same mixed diet. Only animals free from abrasions, lice, vermin and fungi were used. With the help of a binocular loupe and small curved scissors the hairs were cut 1 to 2 days before death, and care was taken not to injure the epidermis. Cutting was performed in a rectangular field (1 cm. × 2 cm.) on each side of the spine in the interscapular regions, leaving a small strip of the coat between the fields. Flaps including the whole skin down to the deep external fascia were then excised. The skin flaps, measuring about 0.7 × 1.5 cm., were fastened with thin steel needles to pieces of cork. To

minimize distortion, faulty stretching and curling of free edges this was done before the last two edges of each flap were cut free. One strip from each cut skin area was then placed for 12 hours in a 4 per cent solution of basic lead acetate (4) and fixed in a mixture of equal parts of formaldehyde solution (14 per cent) and basic lead acetate (8 per cent) for 36 hours. For cytological examination similar flaps were fixed in a solution of formaldehyde, corrosive mercuric chloride and acetic acid. From each flap two sets of paraffin sections were prepared, measuring 4 and 10 μ in thickness respectively. All sections were perpendicular to the skin surface.

All sections of 10 μ were routinely stained in 0.1 per cent toluidine blue solutions in 1 per cent and 30 per cent alcohol (6, 7). The other set of sections was stained for cytological examination. Only sections of 10 μ treated with the basic lead acetate solutions and stained in 0.1 per cent toluidine blue in 30 per cent alcohol solution were accepted for mast cell count.

METHODS OF COUNTING

Mast cell counts in thin sections (10 μ) were done by the following methods:

1. One side of a square-ruled ocular micrometer, (described below,) was placed as close as possible to the borderline between epidermis and dermis. Mast cells were then counted separately in that part of the slide corresponding to the upper half of the micrometer net as well as in that corresponding to the lower half. In other words, the number of mast cells was estimated, in the first instance, in the superficial portion of the dermis, and in the second, in the lower dermis and in the hypodermal tissue (Fig. 1). The slide was then moved laterally and a new pair of half-squares were counted as before. Manipulations and counting procedures were repeated 60 times in each case, and the mast cell count thus obtained gave an average value of the number per tissue volume corresponding to the calibrated square rule. The average numbers derived from the right inter-scapular region of the animals were denoted by A and B respectively, and refer to the upper and lower halves of the micrometer. Correspondingly, the average numbers of mast cells derived from the left side of the animals have been called a and b. Thus,

A and a = the *average* number of mast cells (60 observations) in a piece of the superficial dermis measuring 0.01 mm. $\times$ 0.0044 sq. mm.

B and b = the *average* number of mast cells (60 observations) in a similar piece of deep dermal and hypodermal connective tissue from the left side of the animal.

2. The other method implies that we determine the number of dermal mast cells per 1.0 mm. of epidermal length. This method is designed to avoid the error due to secondary volume increment. Accordingly, a linear calibrated ocular micrometer measuring 1.00 mm. in length is used to apply this standard to the microscopic slides. Thus, 1.00 mm. is plotted with small dots on the slide along the epidermal basement membrane,
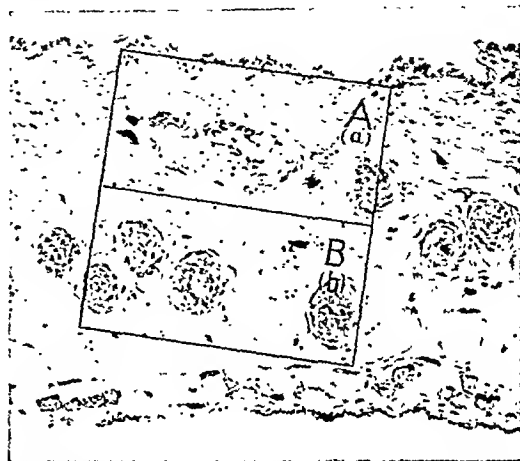
FIG. 1.—Areas for counting of mast cells in normal mouse skin. Mag. $\times$ 125.

and then the number of mast cells is counted in the underlying dermal connective tissue. In this way a total of 40 to 50 mm. of skin is examined in sections 10 μ in thickness. To get such a long area of skin, 6 different non-serial sections have usually been used. The average numbers of mast cells in the dermis per mm. of epidermal length was denoted by C and c corresponding with the designations used above.

C and c = the *average* number of *dermal* mast cells per 1.0 mm. of epidermal length, regardless of dermal thickness. (Sections, 10 μ. Number of observations 40 to 50.)

The following standard microscopic equipment was used throughout: Zeiss achromatic objective No. 20, ocular No. 7, and one ocular, square-ruled micrometer net, measuring 0.3 mm. $\times$ 0.3 mm. (= 0.09 sq. mm.) with reference to the object.

RESULTS

For the estimation of the number of mast cells, the first method was applied to 10 mice 8 weeks old (Table I). Forty-two additional mice 2 to 3 months old were further examined but for the sake of brevity these results are not recorded in detail. However, it may be mentioned that the

number of mast cells in this material showed the same average value (A and $a = 10.3$) and also very large individual variations (Table I). Both methods were used in two groups of litter mates (Table II). Only the second method was applied to 2 groups of litter sucklings (Table III).

The figures in Tables I, II and III and those of the additional material mentioned above justify the following conclusions.

cell count obtained in symmetrical skin areas shows remarkable similarity for both sides as manifested by the reported quotients A/a and C/c. The corresponding quotients B/b do not express as great conformity.

Thus, we have found a method suitable for experimental purposes, *viz.* for studying the effect of different agents on the number of dermal mast cells, provided that symmetrical skin areas from

TABLE I: RELATIVE NUMBER OF MAST CELLS IN THE INTERSCAPULAR SKIN OF NORMAL 8 WEEKS OLD SWISS ALBINO MICE

Mouse No.	Age, weeks	Weight, gm.	Number of mast cells				Quotients	
			Right side		Left side			
			$A \pm E$*	$B \pm E$	$a \pm E$	$b \pm E$	A/a	B/b
1†	8	19.5	10.5±0.5	2.7±0.3	9.9±0.6	3.0±0.3	1.06	0.90
2	8	17.	9.0 0.5	2.5 0.3	9.2 0.6	2.6 0.3	0.98	0.96
3	8	14.5	15.4 0.8	4.2 0.4	16.8 1.0	3.9 0.4	0.92	0.93
4	8	17.5	7.1 0.4	3.3 0.3	6.7 0.4	3.0 0.3	1.06	1.10
5	8	20.	9.4 0.6	2.1 0.2	10.4 0.7	2.4 0.2	0.90	0.89
6	8	18.	19.7 0.9	5.1 0.4	21.5 1.0	5.0 0.4	0.92	1.02
7	8	19.5	8.2 0.4	3.2 0.2	8.4 0.5	3.3 0.3	0.98	0.97
8	8	12.5	11.1 0.7	3.5 0.3	10.0 0.6	3.9 0.3	1.11	0.90
9	8	25.	9.2 0.5	2.1 0.2	8.7 0.5	3.0 0.2	1.06	0.70
10	8	13.5	10.3±0.6	3.8±0.3	10.3±0.6	3.2±0.3	1.00	1.19
Average numbers:			11.0	3.25	11.2	3.33		
Standard deviation for quotients:							±0.08	±0.14

*For explanation of symbols see Methods of Counting.
†Mice Nos. 1 to 5 are litter mates.

TABLE II: RELATIVE NUMBER OF MAST CELLS IN THE INTERSCAPULAR SKIN IN A SECOND SERIES OF 6 TO 8 WEEKS OLD SWISS ALBINO MICE

Mouse No.	Age, weeks	Weight, gm.	Number of mast cells						Quotients		
			Right side			Left side					
			$A \pm E$	$B \pm E$	$C \pm E$	$a \pm E$	$b \pm E$	$c \pm E$	A/a	B/b	C/c
11†	6	19	9.9±0.5	3.8±0.3	40.7±3.5	10.7±0.6	3.9±0.3	42.0±3.5	0.93	0.97	0.97
12	6	16	8.6 0.5	1.8 0.2	28.4 3.0	9.5 0.5	2.2 0.2	27.4 3.1	0.91	0.82	1.03
13	6½	19	7.1 0.4	2.9 0.3	27.2 2.6	7.4 0.4	2.5 0.3	25.0 2.5	0.96	1.16	1.09
14	6½	16	12.8 0.6	3.6 0.3	36.6 3.2	11.9 0.6	3.0 0.3	35.0 3.5	1.08	1.20	1.05
15	7	17	9.6 0.5	3.4 0.3	35.5 3.5	8.4 0.5	3.0 0.3	34.4 3.1	1.14	1.13	1.03
16	7	17	8.0 0.4	2.6 0.3	38.5 4.0	8.7 0.5	2.3 0.2	38.5 3.5	0.92	1.13	1.00
17‡	8	18	10.4 0.6	1.9 0.2	30.3 3.1	11.8 0.6	1.9 0.2	35.1 3.4	0.88	1.00	0.86
18	8	19	10.6 0.6	3.2 0.3	40.2 3.9	10.3 0.6	3.0 0.3	37.5 3.7	1.03	1.07	1.07
19	8½	22	8.4 0.5	1.6 0.2	35.3 3.0	7.5 0.4	1.3 0.2	32.4 3.5	1.12	1.23	1.09
20	8½	18	11.2 0.6	2.5 0.3	43.0 4.2	10.7 0.5	2.0 0.2	38.1 4.0	1.05	1.25	1.13
21	9	18	6.0 0.4	3.6 0.3	28.3 3.0	7.4 0.4	3.4 0.3	30.0 3.2	0.81	1.06	0.94
22	9	18	7.8±0.4	1.8±0.2	30.5±2.8	9.3±0.5	1.9±0.2	34.0±3.1	0.84	0.95	0.90
Average numbers:			A and a =	9.3							
			B and b =	2.6							
			C and c =	34.3							
Standard deviation for quotients:									±0.12	±0.16	±0.09

†Mice Nos. 11 to 16 are litter mates.
‡Mice Nos. 17 to 22 are litter mates.

The *individual variations* of the average numbers of mast cells in the different age groups are very large, both in the dermal (A, a, C, and c) and in the hypodermal (B, and b) material. Even if we could present a "statistically correct" average number of mast cells in mice of different age groups, these figures would be of little or no value to experimental research, because they do not permit any conclusions as to the actual number of mast cells in the individual mouse.

On the other hand, a comparative dermal mast

the same animal always serve as controls. We hardly need repeat that this method so far is applicable only to dorsal skin areas, and is valuable chiefly for studying the strictly dermal mast cells. Furthermore, because of the apparent discrepancy between section thickness and mast cell size, the methods are unsuitable for the estimation of the total numbers of mast cells per tissue unit. If absolute numbers are desired, counting must be performed on thicker sections (*3*).

The number of mast cells was not found to be

constantly larger in young animals (Table III) than in adults (Table II), and thus the statement by earlier investigators could not be corroborated (1, 2). For lack of additional data this fact will not be discussed (3). In most mice high and low numbers of mast cells in the dermis were found to be consistent with respectively high and low numbers of hypodermal mast cells (Table I and II).

TABLE III: RELATIVE NUMBER OF MAST CELLS IN THE INTERSCAPULAR SKIN OF YOUNG ALBINO MICE COUNTED PER MM. OF EPIDERMIS

Mouse No.	Age, days	Weight, gm.	Number of mast cells Right side $C \pm E$	Left side $c \pm E$	Quotient C/c
23	4	3	19.8 ±0.4	19.8 ±0.5	1.00
24	4	3	23.1 ±0.5	23.4 ±0.5	0.99
25	4	3.5	14.7 0.8	16.0 0.9	0.91
26	13	6	17.4 0.8	19.4 0.6	1.12
27	13	5	21.3 0.8	18.0 1.5	1.18
28	13	5.5	20.0 ±1.4	22.2 ±1.2	0.90
Average numbers:			19.1	19.5	
Standard deviation for quotient:					±0.12

STATISTICS AND ERRORS

The errors inherent in both methods presented above depend upon two different types of error, *vis.* (a) biological and individual variations in the frequency of mast cells, and (b) technical errors due to the methods of preparation. Our primary values are influenced by both groups of errors simultaneously. Due to the fact that our methods imply comparisons between the relative numbers of mast cells in symmetrical skin areas from the same animal, we have avoided the error caused by individual variations. The biological variation in the number of mast cells in the same animal is of course not eliminated. We have to mention the following important technical errors: overstretching of flaps, swelling, shrinkage, faulty section angles, and thickness.

A statistical expression of the distribution of our primary values is obtained by calculating the standard error of the mean for A, B, C, a, b, and c, called E_A, E_B, etc. (Eq. 1). The resulting standard errors for the quotients are calculated, (Eq. 2) and amount to $\pm$ 10 per cent for A/a, and $\pm$ 15 per cent for C/c. These standard errors do not include systematic technical errors.

If under extremely unfavorable conditions all sources of error go in the same direction, we would get a considerable total error. But fortunately *the actual error usually is much smaller*, as evidenced in Tables I to III. The standard deviation of quotients A/a and C/c amounts to $\pm$ 10 per cent (Eq. 3). This expression includes all possible sources of error. Thus, we are justified in stating that these counting methods are fairly applicable to experimental purposes.

The methods reported above will be used in serial studies on changes in the frequency of visible dermal mast cells induced by different agents. When these standard methods are applied to frequent serial observations resulting in uniform numerical changes, we accept deviations in quotients exceeding 2σ as evidence of true changes in the number of granule-bearing mast cells.

SUMMARY

In the interscapular skin areas on the dorsum of mice of different age groups, the individual variations in the number of dermal and hypodermal mast cells were found to be so large, that it proved impracticable to determine an average standard number. On the other hand, in each individual the numbers of mast cells were found to be about the same in symmetrical skin areas. Using this fact, two simple methods are described for the quantitative assay of the *relative* number of mast cells in thin tissue sections (10 μ). The methods afford ample possibilities for studying the mast cell reactions to different experimental agents, provided that counts for absolute cell numbers per tissue unit are not attempted.

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Eq. 1
$$E = \pm \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

Eq. 2
$$E_{A/a} = \pm \frac{A}{a} \sqrt{(E_A)^2 + (E_a)^2}$$

Eq. 3
$$\sigma = \pm \sqrt{\frac{\Sigma d^2}{n-1}}$$

The Mast Cell Reaction of Mouse Skin
To Some Organic Chemicals*
II. The Effect of Common Organic Solvents

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In the course of investigations on the metabolism of ester sulphates and mast cells found in the stroma of human tumors (13-16), it was decided to study some principal stroma reactions separately by experimental means. One line deals with the histochemical reactions occurring during the early phases of skin carcinogenesis. With regard to this topic it was felt that careful reinvestigations on the effects of common organic solvents were badly needed. The following report deals mainly with the skin reactions to single painting with some common solvents. Simultaneous changes in the blood, liver, or urine have not been included.

The application of pure *ether* to the skin causes but very slight epidermal swelling (10). Alcoholic solutions have not been tested. No changes have been observed after painting with pure *acetone* (9, 12). On the other hand, pure *benzene* was found to cause swelling of epidermal cell nuclei and cytoplasm (10), a moderate hyperemia (12), followed by inflammatory response on the part of the dermal connective tissue (10), and subsequent epidermal regeneration, characterized by hyperplasia and hyperkeratosis (10). The skin response to repeated benzene painting has been inadequately studied. Orr (9), however, reported a numerical increase of the number of mast cells in the skin of mice 6 to 8 weeks after painting, "almost to as great an extent as with the carcinogens."

A considerable number of workers have lately shown interest in so-called "detoxication mechanisms" induced by organic chemicals. The protective reactions on the part of the skin constituents will be mentioned below with particular emphasis on the work by Crabtree (1, 2).

MORPHOLOGY OF THE DERMAL MAST CELLS

In new-born mice the mast cells of the corium present a fairly uniform ovoid shape and contain a moderate number of specific granules. During the postnatal development of the skin two separate types of mast cells are differentiated. Small mast cells, irregular mast cells poor in granules are seen in the papillary and superficial part of the reticular layer of corium. In the deeper dermis and in the hypodermal connective tissue the mast cells are larger, rounder, and have more granules. In the first type the granules are very small, often dustlike, but in the second type they are coarser. For further morphological details the reader is referred to comprehensive reviews by Lehner (6), Michels (8), and others.

The metachromatic staining reaction of the granular substance (4) is due to their content of sulphuric acid esters (7). This constitutes a characteristic feature of normal mast cells and enables us to distinguish them from other connective tissue cell elements. Normal tissue mast cells do not present any typical fluorescence in ultraviolet light (3, 11). We have to recall that all morphological descriptions refer to dead mast cells treated by various fixatives and other solvents (benzene, alcohol, etc.) and thus devoid of normal lipids.

MATERIAL AND METHODS

All mice used belong to a common Swiss albino stock of mixed genetic constitution, not subjected to inbreeding. They are resistant to the induction of skin tumors. All animals used were free of skin damages, lice and ringworms. Two days before painting, the hairs were cut by hand as described in the preceding paper (5).

Painting was performed with the following pure solvents:

Alcohol 25% solution in distilled water
Ether   pure anesthetic diethyl ether
Acetone pure
Benzene pure acc. Ph. S.

Only the right interscapular region was painted, the symmetrical skin flaps on the left side were used as controls. The animals were killed at daily intervals at the same time, about 10 P.M. One pair of skin strips was cut out for fixation in basic lead acetate, and another pair for fixation in cor-

rosive mercuric chloride solution (5). All technical details concerning the excision of skin flaps, fixation, staining, and preparation of sections were standardized according to methods previously reported (5). Counting of mast cells was undertaken only in isolated (non-serial) sections 10 μ in thickness. Both counting methods described earlier were used (5). In conformity with the foregoing paper we have applied the same statistical methods and demand uniform serial deviations in quotients exceeding $\pm$ 20 per cent as statistical evidence of true changes in the numbers of mast cells (5).

RESULTS

As previously mentioned, only the observations regarding the tissue mast cells will be reported, together with some general tissue changes that are of importance for the interpretation of our findings. Extensive cytological investigations were published by earlier authors.

Alcohol.—A small number of mice 6 weeks old were treated with 12 brush strokes of a 25 per cent solution to the right interscapular region. Skin flaps were taken from both sides 1, 2, 3, 4, 5, and 6 days after the painting. In both the experimental and control skin strips the number of mast cells was found to be of the same magnitude. The quotients showed the following variations:

A/a 0.91 $\pm$ 0.08 to 1.14 $\pm$ 0.09
B/b 0.94 $\pm$ 0.08 to 1.13 $\pm$ 0.14
C/c 0.96 $\pm$ 0.11 to 1.06 $\pm$ 0.14

Standard deviation for quotients was about $\pm$ 10 per cent.

Thus, alcohol painting did not induce conspicuous changes in the number of dermal or hypodermal mast cells within 6 days after painting. Nor were any changes seen in the granular contents.

Ether.—Four full brush strokes of pure ether were applied to the right interscapular region of 15 mice 6 weeks old, and skin flaps, both experimental and control, were examined after 4 hours, daily from the first to the tenth day and on the 12th, 14th, 16th, and 18th days. In all cases the number of mast cells on the experimental side was as great as on the control side, and thus the quotients obtained were about 1. The standard deviation for quotients was similar to that mentioned above. Thus, also in this series there was no effect on the number of mast cells.

No damage to the surface epithelium was observed after the application of ether, and no swelling, thickening or hyperkeratosis could be noted (10).

Acetone.—The effect of pure acetone was studied by painting the right interscapular region of 6 mice 8 weeks old with 3 full brush strokes. Mice were killed 1, 2, 3, 4, 5, and 6 days after painting. The control (left) side was not painted.

Also in this series the same number of mast cells was found in both experimental and control skin flaps. The quotients were about 1.0 and thus, the conclusion was justified that the application of pure acetone did not produce any obvious changes in the number of dermal mast cells.

Apparently, the solvents mentioned above have several characteristics in common. They do not induce conspicuous cell damage, epidermal hyperplasia, noticeable hyperemia or any inflammatory reaction on the part of the dermal connective tissue, at least when used in the reported concentrations and amounts. However, we do not know the amount of resorption of these volatile solvents and their actual effects on the lipid monolayers of the cells. They are of low reactivity and thus do not demand any complicated protective mechanisms on the part of the tissue constituents. Our results indicate that these solvents do not produce any tissue changes involving the granular substance of the mast cells.

Benzene.—The early effects of pure benzene applied to the skin was investigated in two series of mice (Tables I and II, Figs. 1 to 9). Technical details regarding the application of benzene are given under each table. The late effects of single painting with benzene will be reported in a subsequent paper.
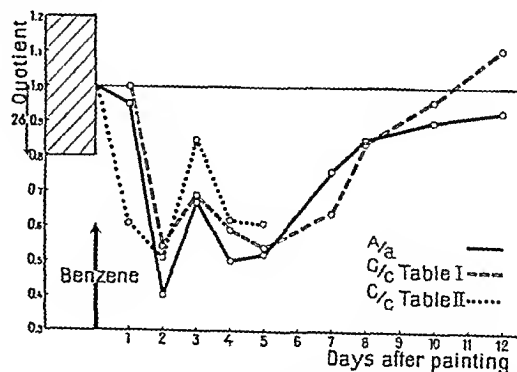
Fig. 1.—Relative number of mast cells in the interscapular skin of mature and young mice after single painting with pure benzene.

Benzene seems to be rapidly absorbed through the intact epidermis. A few minutes after painting many mice show general convulsion of moderate degree. Ten to 15 minutes later they appear, however, to be well again, and remain so until killed.

As to the gross changes a moderate degree of hyperemia was observed in the painted areas during the first 3 days after painting, a fact in accordance with previous statements (12). The microscopic changes were characterized by early swelling of the surface epithelia leading to slight hyperplasia. The dermal edema, described by previous authors, seemed to be moderate and generally more pronounced in younger mice than in mature ones. However, the series are

the cytological changes in the epidermal cells, or to the epidermal regeneration pattern.

In both series of mice the application of pure benzene apparently induced a considerable decrease in the number of dermal mast cells. This decrease is more than twice the standard deviation for quotients (5), and amounted to about 50 per cent during the second to fifth day after painting (Table I). After the fifth day a slow reappearance of the mast cells was observed. The decrease took

TABLE I: Relative Number of Mast Cells in the Interscapular Skin of Mature Mice After Single Painting With Pure Benzene*

No.days after painting	Weight, gm.	Right side			Number of mast cells Left side			Quotients		
		$A \pm E$†	$B \pm E$	$C \pm E$	$a \pm E$	$b \pm E$	$c \pm E$	A/a	B/b	C/c
1	18	5.7±0.3	2.1±0.2	24.1±2.3	6.0±0.3	2.2±0.2	24.0±2.5	0.95	0.95	1.00
2	20	3.4 0.3	1.1 0.2	15.0 1.1	8.6 0.5	1.4 0.2	27.7 3.0	0.40	0.79	0.54
3	16	6.2 0.4	2.7 0.3	20.8 1.9	9.3 0.6	2.4 0.3	30.9 2.8	0.67	1.13	0.69
4	20	6.1 0.4	1.8 0.2	16.0 1.7	12.2 0.7	2.1 0.3	27.0 2.9	0.50	0.86	0.59
5	21	4.7 0.4	1.5 0.2	18.8 2.1	9.0 0.5	2.9 0.3	34.9 3.5	0.52	0.52	0.54
7	18	7.4 0.4	2.5 0.2	31.3 2.8	9.8 0.5	3.4 0.3	48.6 4.5	0.76	0.74	0.64
8	21	5.9 0.3	1.8 0.2	28.6 2.6	7.0 0.4	2.0 0.2	34.2 3.1	0.85	0.90	0.84
10	22	9.0 0.5	2.4 0.3	44.3 4.1	10.0 0.5	2.5 0.3	46.0 4.5	0.90	0.96	0.96
12	17	19.8 0.9	5.2 0.4	83.2 7.8	21.3 0.9	6.1 0.4	75.1 7.2	0.93	0.85	∖1.11
15	22	10.7 0.6	2.7 0.3	49.1 5.1	10.0 0.6	2.6 0.3	54.0 5.1	1.07	1.04	0.91
17	23	9.3 0.5	2.9 0.3	44.9 4.3	9.0 0.5	2.6 0.3	45.3 4.3	1.03	1.12	0.99
22	18	10.0±0.6	3.1±0.3	51.4±4.8	11.0±0.7	2.8±0.3	53.0±5.0	0.91	1.11	0.97
Average numbers for controls:					10.3	2.8	41.7			

*Mixed Swiss albino mice 8 weeks old were painted with 3 full brush strokes of pure benzene on the right interscapular region of the skin, previously cut. The left side remained untreated and served as control.
†For explanation of symbols, *see* Methods of Counting, in the preceding paper.

TABLE II: Relative Number of Mast Cells in the Interscapular Skin of Young Litter Mates After Single Painting With Pure Benzene*

Age, days	No. days after painting	Weight, gm.	Number of Mast Cells Right side $C \pm E$†	Left side $c \pm E$	Quotients $C/c \pm E$
14	1	4.5	23.6±2.9	39.0±1.6	0.61±0.08
15	2	4.8	18.5 2.8	36.0 5.0	0.51 0.10
16	3	6.0	60.1 2.1	70.9 4.6	0.85 0.07
17	4	6.0	32.7 1.8	52.6 2.0	0.62 0.04
18	5	6.0	33.2±2.1	54.4±2.3	0.61±0.04
Average number for controls:				50.6	

*Litter mates 13 days old were painted with 12 full brush strokes of pure benzene on the right interscapular region of the skin, previously cut. The left side remained untreated and served as control.
†For explanation of symbols *see* Methods of Counting, in the preceding paper.

not comparable in this respect because of difference in dosage. Previous statements by Orr (9), and Stowell and Cramer (12) concerning the inflammatory dermal reaction have been verified. Thus, a *slight*, chiefly neutrophilic cell infiltration was found in the dermal connective tissue, but only in places and never throughout the dermis. The cell infiltration was most striking on the second to fifth days after application, then decreased. Neither the edema nor the inflammatory cell infiltration were considerable and did not produce any observable changes in texture of the loose connective tissue. No special attention was paid to

place chiefly in the mast cells of the superficial and middle part of the dermis, leaving the hypodermal mast cells comparatively unchanged, as may be seen from the quotient B/b. As mentioned in the preceding paper (5), the second method for counting mast cells excludes the influence of uncontrollable volume increments due to edema and inflammation. When this method was applied to a series of young mice consistent results were obtained (Table II and Fig. 1).

The interpretation is quite clear. We are not justified in believing that the dermal mast cells disappeared from the tissue in question. Instead, they become temporarily invisible. About half of them have lost their specific granules and are no longer stainable with the metachromatic dye. After several days the granular substance gradually resynthesized and thus a number of mast cells reappear in the dermis. These events are amply demonstrated by the observation that the granular content of the superficial mast cells during the first and second days after painting decreases. On the other hand, the reverse observation was established during the fifth to 15th days after painting.

The question, then, is raised as to what happens to the granular substance of the superficial mast
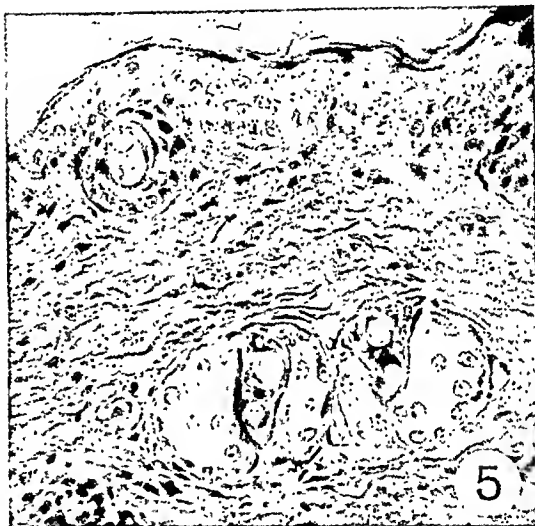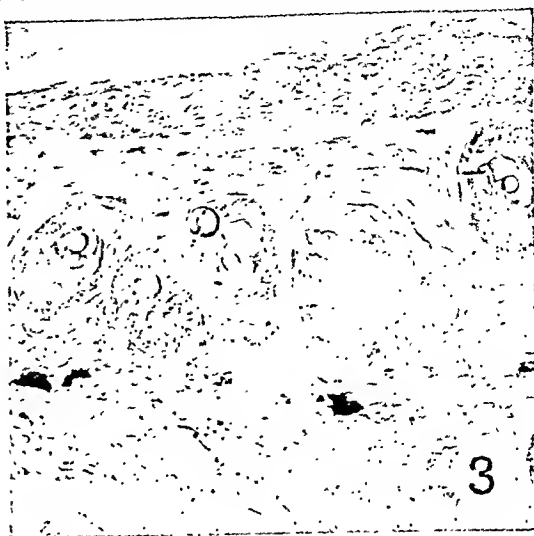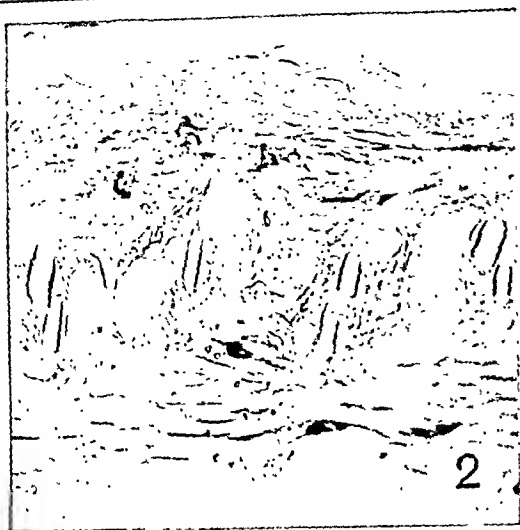
FIG. 2.—Control skin area in mature mouse with normal distribution of mast cells. Toluidine blue. Mag. × 325.

FIG. 3.—The opposite skin area of the same mouse 3 days after one single application of pure benzene. Marked depletion of superficial dermal mast cells. Toluidine blue stain. Mag. × 325.

FIG. 4.—Same as Fig. 2. Van Gieson stain. Mag. × 325.

FIG. 5.—Same as Fig. 3. The cytological changes of epidermis and the moderate inflammatory dermal alterations are seen. Van Gieson stain. Mag. × 325.

cells. In this series of experiments no signs of metachromatic substances being liberated from the mast cell cytoplasm were seen; no "free chromotrope substances" (14, 16) were found in the dermal connective tissue or in the epidermis coincidental with the loss of granular substance from mast cells; no metachromatic inclusions in connective tissue cell elements or macrophages could be discerned. Nor did the intercellular fluid contain any substances presenting a true metachromatic staining reaction. This was checked by the freezing-drying technic in a small number of cases. In other words, the present investigation could not elucidate the fate of the liberated granular substance.

It may be well to stress that the decrease observed in the mast cell number was strictly limited to the painted skin areas. In the control flaps on the left side of the animals the numbers of mast cells were as great as in unpainted mice (5).

Concerning the quantitative correlation between dosage and the resulting loss of granular substance, these investigations are inconclusive. The maximal loss of visible mast cells for both series is about 50 per cent. Considering our deficient knowledge of the amount of granular
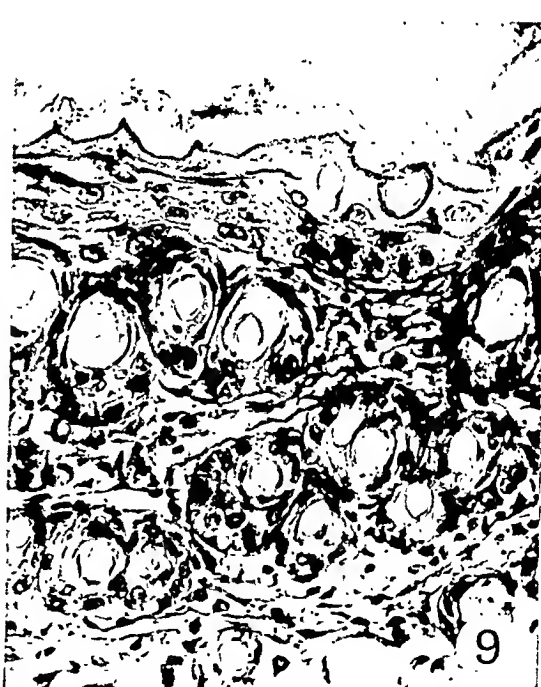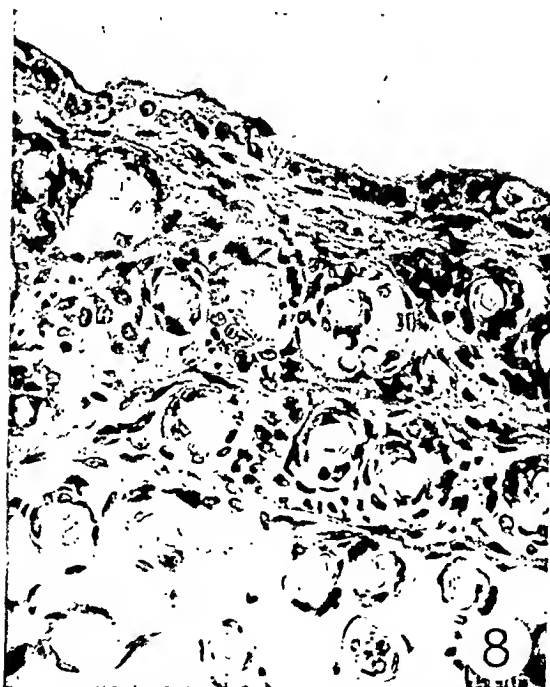
FIG. 6.—Unpainted control skin area from 13 days old mouse. A considerable number of mast cells in the superficial part of dermis. Toluidine blue stain. Mag. × 325.

FIG. 7.—Two days after painting with 12 brush strokes of pure benzene on the right interscapular skin a very marked depletion of mast cell granular substance is seen. Same animal as in Fig. 6. Toluidine blue stain. Mag. × 325.

FIG. 8.—Same as Fig. 6. Van Gieson stain. Mag. × 325.

FIG. 9.—Same as Fig. 7. Pronounced epithelial changes and moderate inflammatory cell reactions on the part of the dermal connective tissue. Van Gieson stain. Mag. × 325.

substance in the mast cells we are not justified in making any definite statements as to the quantitative correlations before comparable data are obtained. These conditions will be further discussed in a subsequent paper.

With a view to the quotient B/b, which refers to the deepest dermal and the hypodermal layers, we have to emphasize its usual instability. Even in normal mice this quotient showed very large individual variations (5), and so we are compelled to state that the values obtained in this series render no simple conclusions possible. The suggestion seems justified that mast cells situated in the hypodermis do not participate in the reaction to benzene painting, at least not under the conditions used in this experiment. Hypodermal mast cells are not included in the count for each millimeter of epidermal length (Tables I and II: C and c).

As far as we know, this early effect of single applications of pure benzene to the skin has been neither observed before nor predicted. With a view to continued research on the biological significance of the granular substance in mast cells and the possible types of reactions in which the constituents of the granules may take part (13-16), it seems to be of importance to consider the nature of the reaction reported above. The suggestion will thus be advanced, that the immediate response to benzene painting is a local depletion of labile sulphur compounds in the skin (1, 2). This depletion is an early reaction, occurring during the first hours after painting, and it may secondarily induce a subsequent series of reactions tending toward the restoration of the sulphur level. The granular substance of the tissue mast cells might achieve this sulphur restoration in the surrounding connective tissue, and these cells might have a role to play in the production of sulphurous compounds to the tissues. These results and their possible significance open new doors to the approach of the mast cell problem, and it seems advisable to undertake more careful studies on the reactions of skin to different chemicals. Further discussions will therefore be postponed until additional data can be presented.

SUMMARY

The morphological response on the part of the tissue mast cells to single applications of some common organic solvents has been investigated in the skin of mixed albino mice. Serial observations of the number of dermal and hypodermal mast cells, together with approximate estimations of their granular content are reported. Chemically inert solvents such as alcohol, ether, and acetone did not induce any significant changes. On the other hand, single paintings with pure benzene resulted in depletion of the metachromatic granular substance of the superficial mast cells. This effect was strictly confined to painted skin areas, and was found to occur late during the first day after painting and to reach a maximum level during the second to fifth day after painting. The suggestion has been advanced that the granular substance is delivered to the skin in order to restore its content of labile sulphurous compounds, which have been depleted during the first hours after painting (1, 2).

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Mast Cells in Experimental Rat Sarcomas*

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The mast cells described by Ehrlich (6) are characterized by granules showing a so-called true metachromasia. Using as a basis Lison's statement (22) that this color reaction is typical of macromolecular sulphuric ester compounds and Jorpes' (17) demonstration that heparin is a mucoitin ester sulphuric acid showing a distinct metachromasia, Holmgren and Wilander (11) proved the mast cell granules to consist of a material with the same properties as heparin. These authors were able to isolate 22 mgm. of active heparin from 10 gm. of mast-cell-rich capsule of cow liver, whereas preparations of mast-cell-poor capsule of lamb liver proved to be inactive. Jorpes, Holmgren and Wilander (19) also confirmed the conception of mast cells as bearers of mucoitin ester sulphuric acid (heparin). Later this was corroborated by Hirth (10). Wilander (33) in a more comprehensive treatise on the nature of heparin demonstrated the mast cells to be the bearers of heparin. Mast cells are found exclusively in connective tissue, in rather variable amounts within the different parts of the body. Ehrlich had already emphasized that these cells occur chiefly in the vicinity of blood vessels. For bibliographical notes pertaining to mast cells, see Lehner (20), Holmgren and Wilander (11) and Michels (25).

The pathology of mast cells seems still to be only partially recognized. Maximow (24) states that mast cells change their appearance during inflammation. Some are said to burst, and their granules are found in the tissues. Maximow also emphasizes that the new tissue is free from mast cells. Nakashima agrees with Maximow and stresses the fact that during the degeneration of mast cells the granules lose their metachromasia. In addition, Ernst (8) describes morphologic changes of mast cells during the first hours of the inflammatory process.

The appearance of mast cells in tumors of various kinds has been dealt with by many authors. Sylvén (31) described the appearance of mast cells in sarcoma of connective tissue origin. He emphasized that the largest number of these cells has "always been demonstrated within the peripheral parts of the tumors, where the infiltrative destructive growth and disintegration of surrounding normal tissues takes place." On the other hand, mast cells occur only occasionally in the central parts of the tumor. Sylvén (31) has reviewed the literature on mast cells in mesenchymal tumors.

The fact that several authors (1, 3-5, 9, 21, 26, 28, 29, 32) have pointed out the large numbers of mast cells to be found in the skin of mice with tar cancer is of considerable interest for our work. Mast cells will sometimes even form proper nevi (3, 4, 9, 29). Borrel, Boez and de Coulon, (3) who were of the opinion that cancer is caused by a virus, thought that the mast cell reaction "opened the door to all kinds of infections and thus favoured the development of cancer," whereas Cramer and Simpson (4) believed the accumulation of mast cells to be a defensive process directed against the development of cutaneous cancer.

Cramer and Simpson (4) carried out a thorough investigation of the appearance of mast cells during the development of skin cancer after application of a 0.6 per cent solution of methylcholanthrene in benzene to the dorsal skin of mice. They demonstrated the accumulation of mast cells as a rule to be proportional to the epidermal hyperplasia and considerably to precede the development of malignant growth. In the tumor itself mast cells are scarce but are found in large masses in the immediate neighborhood of the growth, especially where the adjacent epidermis shows advanced hyperplasia. Bloom (2) described the spontaneous appearance of tumor-like masses of mast cells in the skin of older dogs without skin cancer. Cramer and Simpson (4) pointed out that this observation is of interest, as skin cancer is relatively common in dogs.

With regard to human pathology, the appearance of large masses of mast cells in lesions of a fairly rare skin disease, e.g. urticaria pigmentosa, should be mentioned. No relationship of this disease to skin cancer is known.

Finally the fact may be recalled that human myeloid leukemia is characterized by an increased number of blood-mast cells, as described by Holmgren and Wohlfart (14). The relative percentage of blood-mast cells may sometimes rise to about

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40 per cent and higher in so-called mast cell leukemia.

In investigations on experimental sarcoma in rats it was noted that the tumors often contained large amounts of mast cells. The results of a study of this subject are presented in this paper.

MATERIALS AND METHODS

In the investigations to be described white rats from a strain bred at the Karolinska Institute were used. At the beginning of the experiments the animals were usually from 2 to 3 months old. In order to produce sarcoma experimentally, one of the following methods was adopted:

I. Subcutaneous implantation of small pieces of 4 per cent methylcholanthrene-cholesterol into the back.

II. Single subcutaneous injection of about 0.1 cc. of a 0.5 per cent olive oil solution of methylcholanthrene into the back.

III. Single subcutaneous injection of about 0.1 cc. of a 0.5 per cent 3,4-benzpyrene olive oil solution into the back.

Irrespective of the method used, after 5 to 10 months a localized tumor was often found, which proved histologically to be a sarcoma, although of varying type. In the transplantation experiments small pieces of tumor were implanted subcutaneously into the back by means of a trocar. These transplants often "took" quite easily. In some cases, however, the result was somewhat less satisfactory depending on the kind of the transplanted tumor pieces. All the specimens for histological examination were fixed with 10 per cent formaldehyde solution. In some cases 4 per cent basic lead acetate was used, an appropriate fixation for mast cell granules in view of their possible water-solubility (11, 12). When dealing with mast cells of rats, formaldehyde is used with advantage, as the granules hardly dissolve in water. The diffuse tissue metachromasia seems to be more susceptible to this influence, which is counterbalanced by the use of lead acetate. The specimens were embedded as usual and cut in sections 5 to 10 μ thick. The sections were stained with 0.5 per cent toluidine blue aqueous solution. Details of fixation and staining have been described by Holmgren (12) and Sylvén (31)

RESULTS

A total of 47 cases of sarcomas experimentally produced in white rats was studied. In 27 cases these sarcomas were produced by injections of methylcholanthrene, in 16 cases by implantation of pieces of methylcholanthrene-cholesterol, and in 4 cases by injection of benzpyrene. All 3 methods resulted in sarcomas of varying types.

The majority of the tumors were of polymorphocellular or fibroblastic character. Frequently the type of tumor changed in different portions. In a small number of cases lipomyxomatous and rhabdomyosarcomatous types were observed. When stained with toluidine blue, cells containing metachromatic granules, *i.e.* mast cells, appeared in varying number in all tumors, in the tumor tissue as well as in the capsule. While mast cells occurred rather infrequently or were almost lacking in the polymorphocellular tumor portions (sometimes there were none in several visual fields), they were usually present in large numbers, and sometimes abundantly in the fibroplastic portions (Fig. 1). In the rather infrequently observed lipomyxomatous portions only occasional mast cells were found, whereas their number was somewhat larger in the rhabdomyosarcomatous portions.

In Figs. 1, 2 and 3 the varying mast cell incidence in fibroblastic sarcoma portions will be seen. Definite interrelation with the intratumoral blood vessels was not noted. Neither did there emerge a connection between the degree of vascularization and the percentage of mast cells. The polymorphocellular sarcomas were, by the way, extraordinarily vascularized.

As a rule there was no morphologic difference between the mast cells within the tumors and those situated in other tissues. The content of granules in these cells varied greatly (Fig. 4). Within the frequently occurring necrotic areas, uninjured mast cells were sometimes seen (Fig. 5). This might be taken to indicate a greater resistance of mast cells than of other cells. If mast cells are amoeboid cells, as Lehner (20) and others assume, one might suppose that they invade secondarily the necrotic parts. However, the fact that mast cells as a rule are regularly scattered over the necrotic areas and generally quite as thickly as in the neighboring portions, fails to support this presumption. Sometimes the mast cells situated within the necrotic areas displayed a varying affinity to the stain, so that the granules stained a dark blue or blue-green with toluidine blue. In the most advanced necroses mast cells have completely disappeared or are noticed only as indistinct, considerably degenerated cells. The mast cells with blue to blue-green granules are also found in the normal tumor tissue, although more sparcely. One might blame deficient staining, yet this cannot obtain, since around the cells in question other cells are found with obviously metachromatic granules. According to unpublished observations of Holmgren, these cells occur also under other similar conditions. They are to be regarded as having undergone certain changes.
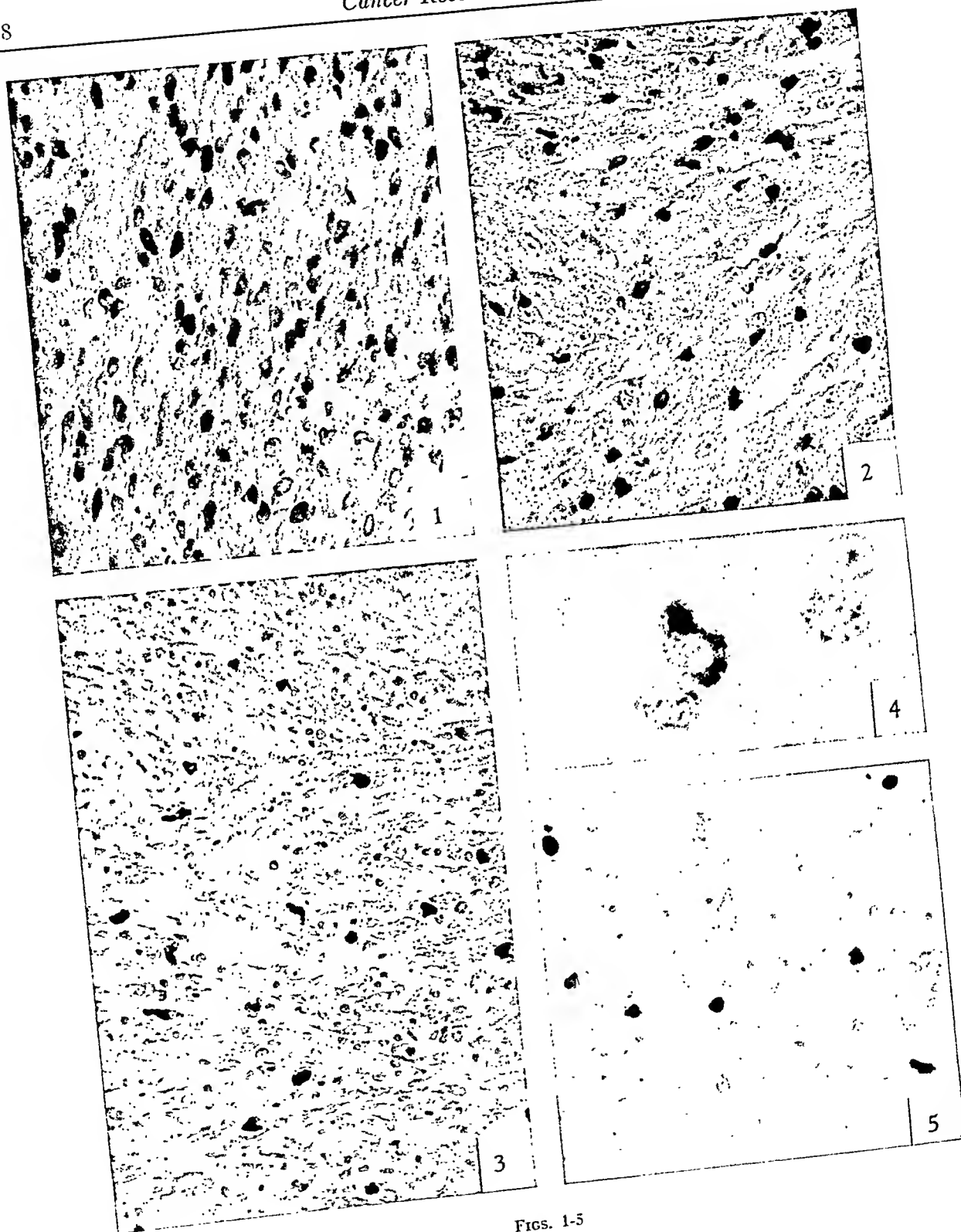
FIGS. 1-5

Diffuse tissue metachromasia was often noted within the tumors. The same type of diffuse metachromasia has previously been described by Holmgren (13) and others in growing embryonal tissue and was recently found by Holmgren and Rexed (15) in the Büngner bands of regenerating peripheral nerves in rats. Sylvén (31) observed in granulation tissue and in various mesenchymal tumors diffuse metachromasia which he called "free chromotrope substance" and which in his opinion is due to mast cells. This conception is based upon a subjective estimation of the quantity of granules and the number of mast cells in tissue containing much or little free chromotrope substance respectively. Sylvén states that fewer mast cells, poor in granules, are present in the former than in the latter. It is of interest to note that Quensel (27) found in cancer a "mucoid" which stains similarly to mast cells, wherefore he presumes it in both cases to be the same substance.

In this investigation no very close attention was paid to the diffuse tissue metachromasia. As for Sylvén's theory, the fact should be stressed that no obvious connection, whatever, between diffuse tissue metachromasia and mast cells content emerged in our material. One could, for example find quite often that areas with strong diffuse metachromasia also contained a large amount of mast cells. These cells, with variable content of granules appear also in parts without diffuse metachromasia.

The enormous number of mast cells that we found in some tumors raises the question of the histogenesis of mast cells. One might ask oneself whether these cells invaded from adjacent structures or originated from the stroma of the tumor tissue. The experimental sarcomas may grow to an exceedingly large size (in our series we met with tumors weighing over 200 gm.). If these tumors are extremely rich in mast cells, as is quite often the case, the neighboring tissue could be expected to be strikingly poor in mast cells, provided the mast cells invaded the tumor from the vicinity. Examination of the tissues surrounding the tumors, however, always showed a fairly normal

mast cell content. In no case did a rough subjective estimation of the mast cell content reveal a difference as compared with normal animals. As already mentioned, the tumor capsules contained a remarkable amount of mast cells both in fibroplastic and polymorphocellular sarcoma. *A priori* the most likely assumption appears to be that the mast cells develop locally in the connective tissue of tumors. The fact that part of the mast cells contain only a few metachromatic granules might also be taken to suggest such an origin. Under these circumstances one would expect to find evidence of mitosis. However, in spite of systematic investigation in no case were signs found indicating mitosis or amitosis. This agrees with the fact that neither Holmgren (15) or others have found mast cells in a state of mitotic or amitotic division in fetuses of rats, mice or man. The appearance of mast cells with only a few metachromatic granules might naturally indicate that these cells had lost most of their granules, and it would be difficult to exclude this possibility in every case. The conception that the granule-poor cells in tumors rich in mast cells might represent young cells where the granules are developing, is supported by a certain similarity of these cells with mast cells in fetuses. There one also finds that the granules appear first in the peripheral parts of the cells as small, sometimes dust-like particles.

In one experiment macroscopically healthy normal tumor pieces were under aseptic conditions transplanted subcutaneously into 10 to 15 young rats. Nearly one-half of the transplanted pieces started growing. From one of the daughter growths new transplantations were carried out in the same way. Thus we followed a tumor through 4 generations, not including the mother growth. Both the mother growth and the various "generations" appeared histologically to be fibrosarcomas. The mother growth contained relatively few mast cells. The first tumor generations presented the same picture as the mother growth and also contained few mast cells. The tumors of the last two generations showed more polymorphous cells and

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DESCRIPTION OF FIGURES 1 TO 5

FIG. 1.—Fibrosarcoma from white rat, extremely rich in mast cells, and induced by subcutaneous injection of 3, 4-benzpyrene in olive oil. Toluidine blue stain. Mag. × 250 (approx.).

FIG. 2.—Sarcoma rich in mast cells from white rat induced by subcutaneous injection of methylcholanthrene in olive oil. Toluidine blue stain. Mag. × 400 (approx.).

FIG. 3.—Sarcoma from white rat with fair number of mast cells, induced by subcutaneous injection of methyl-

cholanthrene in olive oil. Toluidine blue stain. Mag. × 250 (approx.).

FIG. 4.—Higher magnification of mast cells in sarcoma from white rat, induced by methylcholanthrene in olive oil. Granulation varies greatly. Toluidine blue stain. Mag. × 1,200 (approx.).

FIG. 5.—Mast cells in necrotic area of sarcoma, produced in white rat by subcutaneous injection of methylcholanthrene in olive oil. Toluidine blue stain. Mag. × 250 (approx.).

more necroses than the first generations. Quite naturally it was difficult to determine the exact number of mast cells owing to the varying distribution in the different areas. Therefore, we always examined several portions of the tumors. On the whole we received the impression that with every new generation the picture became more polymorphous, the number of mast cells simultaneously declining. Other transplantation experiments were carried out only through 2 daughter generations. Nothing of interest emerged in addition to these findings.

From our experiments it follows that mast cells consistently occur in experimental sarcomas of white rats, caused by carcinogenic substances, and especially in those of the fibroblastic type, where they sometimes even reach an excessive development. It is of interest that the mast cells accumulate to a certain extent in the tumor capsules encasing the sarcomas, and in the connective tissue beneath the skin hyperplasias and carcinomas artificially produced by Cramer and Simpson (4). The study of tumors by Sylvén (31) showed that the number of mast cells in his 21 cases of sarcomas of connective tissue origin varied but that the largest amount was found in the periphery of the tumors where "the infiltrative-destructive growth and disintegration of surrounding normal tissues takes place." According to Sylvén, as a rule mast cells are occasionally found in the central parts of the tumors, especially along the vessels. The content of granules in mast cells within the infiltration zone varies widely, and it is not unusual to find only a few granules.

Of course, it is not possible to explain the cause of the high incidence of mast cells in certain experimental sarcomas. One feels rather inclined to the belief that these cells take part in the reaction of the system against tumor cells. If this is the case, however, various types of sarcoma seem to produce a changing reaction in the system. The question whether the varying mast cell content depends upon the degree of differentiation in the various tumor types or upon other factors, must be left in abeyance. The assertion by Brack (3a) that rapidly-growing tumors in the epidermis and in the alimentary canal should contain a large number of mast cells is of interest. It is possible that the organ or tissue in which the tumor grows has some influence on the amount of mast cells in the tumor. Furthermore, the position is complicated by the fact that in some tumors there are alternately areas rich and poor in mast cells without any other difference in their histological appearance. At any rate, the appearance of masses of

mast cells seems to characterize certain experimental tumor types. The established fact that mast cell granules consist of the anticoagulant heparin is probably of significance in explaining their appearance in tumors.

The statement of Cramer and Simpson (4) that fixation in formalin dissolves the mast cell granules in rats, resulting in the appearance of granule-poor mast cells is of interest in this connection. Fixation in alcoholformol has not the same effect. In our cases we used lead acetate in addition to formalin. The former coagulates heparin and preserves the mast cell granules in rabbits where they are extremely soluble in other fixation media. In our specimens fixed with lead acetate, we observed the same pictures as in those treated with formalin. Therefore, it does not seem likely that the mast cell granules, which in rats are very resistant to water, can be dissolved (12). The inference of Cramer and Simpson (4), that in rats mast cell granules occur in a water-soluble and an insoluble form, does not seem to be satisfactorily established. Furthermore, the authors state that a powerful mast cell reaction develops after treatment with methylcholanthrene and that "certain groups of mast cells show a strong golden-brown fluorescence." According to others and from our own experience, normal mast cells possess no auto-fluorescence. Since methylcholanthrene, which was applied to the skin, has an auto-fluorescence, it appears more likely that the fluorescent cells observed by Cramer and Simpson (4) were macrophages loaded with substance. Admittedly, the fluorescence of methylcholanthrene is bluish, but we do not know whether this color is changed by the fixation. This possibility was disregarded by Cramer and Simpson (4). In addition, F. Sjöstrand (30) found the macrophages to emit a pronounced fluorescence, a fact that should be borne in mind in this connection. This fact should be considered before accepting the statement of Cramer and Simpson as to the auto-fluorescence of mast cells under the conditions described by them.

SUMMARY

Experimental sarcomas in white rats produced by carcinogenic substances regularly contain mast cells. In such sarcomas of fibroplastic type the development of mast cells can attain an extreme degree.

The mast cells present in experimental sarcomas seem to develop locally in the connective tissue of the tumors, and this suggests that they take part in the reaction of the system against the tumor cells.

Within the necrotic tumor areas the mast cells

are frequently well preserved, a fact that seems to point to these cells possessing a greater power of resistance than the tumor cells.

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Pigmented Precancerous and Cancerous Changes in the Skin

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The changes to be described here concern pigmented Bowen's disease, squamous-cell carcinoma and basal-cell carcinoma of skin. They do not include the group of melanoma, melano-epithelioma or melano-carcinoma, nor the pigmentation in conditions sometimes leading to cancer, such as, senile keratosis, keratosis resulting from arsenic, tar or radiation and xeroderma pigmentosum. The changes described in this paper have not attracted enough attention of dermatologists, and Eller and Anderson (8) have stated that pigmented basal cell carcinomas were "quite uncommon." At a recent symposium on "Malignant

The present study is based on tumors from 15 patients seen by us during the last 5 years at the Tata Memorial Hospital. Four of them showed multiple lesions, and will be considered separately from the rest. The following table summarises information regarding age, sex, location etc. in the remaining 7 out of the first group of 11 cases. These tumors were all deeply pigmented and histologically presented the structure of a basal-cell or a basal-squamous type of carcinoma. They were similar in so many features, that only 4 out of 11 have been described as illustrating their probable mode of evolution.

Case No.	Nationality	Age	Sex	Site	Duration	Diagnosis
1	Muslim	45	M	Chin	1 year	Basal sq. cell ca
2	Parsee	60	M	Scalp	5 years	Basal cell ca
3	Hindu (Deccani)	38	M	Leg	Mole since childhood, recent growth 3 weeks	Basal cell ca
4	Hindu (Gujarati)	54	M	Forehead	8 years	Basal cell ca
5	Parsee	73	M	Nasolabial fold	4 years	Basal sq. cell ca
6	Parsee	59	F	Arm	6 months pigmented mole started bleeding	Basal cell ca
7	European	50	F	Forehead	2 years	Basal sq. cell ca

Melanomata" in Leeds, England (4) the opinion was expressed that, "It was surprising but true, that these (pigmented tumors of epidermal origin) are not recognised by the majority of pathologists, and there is very little in the literature about them. But it is very important that they should be recognised, for the prognosis and treatment in these cases is exactly similar to that of the non-pigmented tumors of the same series and quite different from that of the melanomata. The important point is for the pathologists to recognise that pigment formation is a function of a group of tumors other than the true melanomata". It has been suggested that many melanotic tumors reported to have been cured by local excision or radiation were probably tumors belonging to this group. It is known that some of these tumors originate from pigmented nevi and without a histological examination are not easily distinguished from true melanomas.

REPORT OF CASES

Case 8. #13052.—A 64 year old lean Parsee saw a skin specialist for a fungus infection of his feet and one finger. He also casually referred to an "eruption" on the chest which was diagnosed as a "precancerous condition." The patient was therefore referred to the Tata Memorial Hospital. On examination a roughly oval skin lesion was seen over the right lower ribs in the nipple line. It had started as a small dark spot several years back and had gradually increased in a circular spreading manner. There was neither pain nor itching but a slight oozing of clear fluid from the surface. The lesion was about 5 cm. in diameter. Its edge was raised, about 1 mm. wide, wavy and dark purple in color. It was clearly demarcated from the adjacent normal skin. The central portion was smooth, glazed and pale pink. A cluster of few raised pigmented spots was seen in the center. A biopsy from the edge showed the characters de-
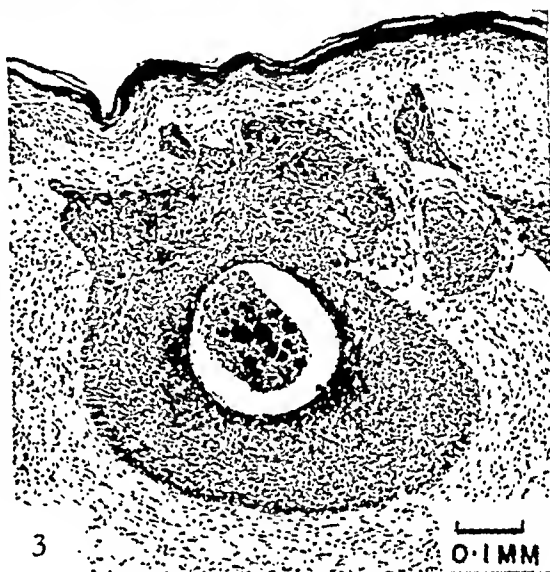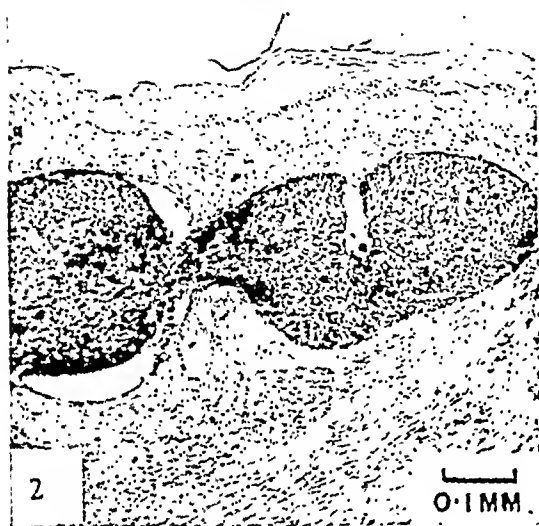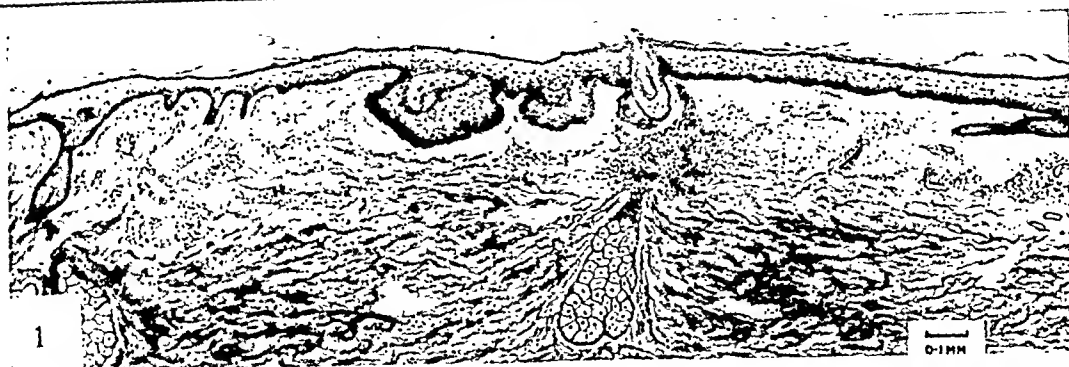
Fig. 1.—Case 8.: A camera lucida drawing of a section of the entire tissue removed by biopsy. The oval bud in the center is over the spreading edge of the lesion; and the healed atrophic skin is seen towards its right. Masson's trichrome stain. Mag. × 65.

Fig. 2.—Case 8.: Photomicrograph showing a network of fine argentophil fibers formed by the numerous dendritic processes of proliferated melanoblasts in epithelial buds. Silver impregnation. Mag. × 100.

Fig. 3.—Case 9.: Photomicrograph showing cords of proliferating epithelial cells pushing into the dermis. The central bud shows an area of degeneration and necrosis in the middle with a debris of pigment granules and desquamated cells. Hematoxylin and eosin stain. Mag. × 100.

scribed below. The lesion regressed rapidly and completely with four exposures of 600 r units daily (85 kv; 1 Al; T. S. D. 15 cms. and a total radiation of 2400 r).

Histological examination.—The biopsy piece (Fig. 1) consisted of stratified squamous epithelium clothing a fibrovascular layer of dermis. The epidermis was thinner than the normal for that region and showed towards the middle of the section a small oval bud of proliferated basal cells pushing downwards in the dermis. The peripheral cells were columnar, contained large oval nuclei and a small amount of pale basophilic cytoplasm. These cells were arranged in a palisade. The more loosely arranged central cells were smaller with round or oval nuclei containing sparse granules of

chromatin material. There was a minute area of surface ulceration over the epithelial bud. The epidermis of the normal skin showed widely separated short rete cones. The basal layer of cells was darkly pigmented with fine brown particles. In the healed area, towards the center of the lesion, there was a complete obliteration of rete cones and the epithelium was flat and thin. The basal cells were without pigment. In this area the pars papillaris of the dermis showed a loose, irregular texture of collagen fibrils, interspersed with arcades of newly formed blood capillaries and foci of mononuclear cellular exudate. The pilosebaceous structures were distorted and atrophic. The downgrowing bud was lying in a bed of concentrically arranged lax, edematous collagen

fibrils. Focal accumulations of lymphocytes and histiocytes were lying outside this zone. In sections impregnated with silver the epithelial bud showed the proliferating basal cells interwoven with a large number of melanoblasts connected with a meshwork of fine argentophile fibers (Fig. 2) formed by the numerous dendritic processes of these cells. The elastic tissue net was absent in the superficial zone of the dermis except along the few hair sheaths and ducts of sweat glands. There were few macrophage cells loaded with coarse brown pigment in the dermis. The section gave an impression of healing at the center and a spreading neoplastic edge at the periphery, consisting of proliferated basal cells and melanoblasts. In the healed area the epidermis and the surface zone of the dermis were morphologically altered, but no trace had been left of the neoplastic epidermal cells.

Case 9. #14275.—A fair complexioned Parsee, 47 years old, had two small pigmented moles on his body "ever since he could remember." One was situated over the middle of the right clavicle and the other in front of the upper third of the right arm. The latter was slightly raised above the surface, about the size of a lentil (5 mm.) and surrounded by an areola of brownish skin roughly 2.5 cm. broad. About eight years back the mole on the arm began to grow in size and the surface "broke into scab covered black fragments". Recently the fragments began to itch and weep. He consulted a surgeon who excised the two moles and sent the one from the arm to us for histological investigation.

Microscopic examination showed a picture similar to that of the advancing edge in Case 1, except that there were several separate buds of proliferating basal cells growing deeper down into the dermis. The bigger buds showed a central area of degeneration and necrosis, with a clear space containing a debris of pigment granules and desquamated flakes (Fig. 3). The basal cells tended to flatten as they approached the core. There was no evidence of healing. The melanoblasts appeared to proliferate and migrate away from the periphery of the buds. They were gradually involved in the degenerative process of the cells towards the center. The proliferating buds of epidermal tissue lay in a broad sheath of loosely arranged collagen fibrils. Subjacent to the superficially ulcerated epithelium, there was a rich cellular exudate of lymphocytes, eosinophiles and histiocytes, between richly sprouting blood capillaries.

Case 10. #E788.—An olive-complexioned 63 year old Anglo-Indian physician had a small hairless mole on his right forearm lateral to the flexor tendons, about 6 cms. above the wrist for "many, many years." The color of the mole was uniformly black and it was smooth on the surface. About two months back he noticed that the mole had begun to increase in size and to itch. The physician attributed this to long hours spent every day in filling a multitude of army forms. He remembered that the itching sensation began one night and the next morning there was a slight erythematous area round the mole. The surface became rough and raised with a couple of weeping points exuding a clear pinkish fluid. No scabs were formed. As the mole rapidly doubled its size, the physician consulted a surgeon who excised the mole and gave him a rather gloomy prognosis about his condition. He saw us with his excised tissue.

A naked-eye examination of the tissue showed a superficially ulcerated, small (6 mm.) brown nodule raised about 3 mm. above the surface. On cut section an ovoid, dark brown, firm mass clearly stood out from the dermis. It was darker near its outer edges. The microscopic examination revealed a sharply circumscribed nodule of neoplastic cells (Fig. 4), composed of lobules separated by filamentous processes of fibrovascular connective tissue. The cords and lobes presented the characters of a baso-squamous epithelioma (Fig. 5) with round or oval spaces in the center filled with fine concentrically arranged lamellae of keratinized material. An area of surface ulceration was dipping into dilated follicles, crypts and fissures burrowing into the tumor mass. The tumor cells in the peripheral cords were unevenly laden with dark brownish pigment. The silver preparation (Fig. 6) showed a proliferation of melano-

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DESCRIPTION OF FIGURES 4 TO 7

FIG. 4.—Case 10.: A camera lucida drawing of a section of the nodule removed by operation. Faintly stained with hematoxylin. Mag. × 25.

FIG. 5.—Case 10.: Low power photomicrograph of the area marked in Fig. 4 above, showing the characters of a baso-squamous epithelioma. Hematoxylin and eosin stain. Mag. × 150.

FIG. 6.—Case 10.: Low power photomicrograph of a portion of the area marked in Fig. 4 above, showing a proliferation of melanoblasts, with a network composed of their branching protoplasmic processes. Silver impregnation. Mag. × 200.

FIG. 7.—Case 11.: Photograph showing a superficially ulcerated dark nodule on the face.

10 MM

0·1MM. 5

0·1 MM. 6

7
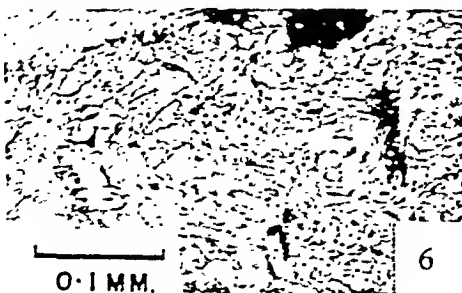
Figs. 4-7
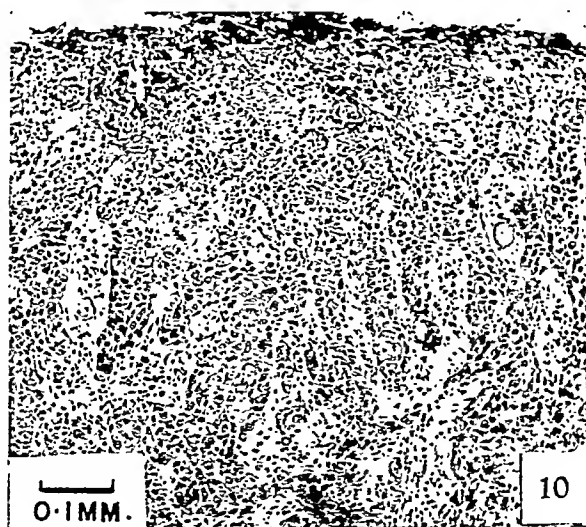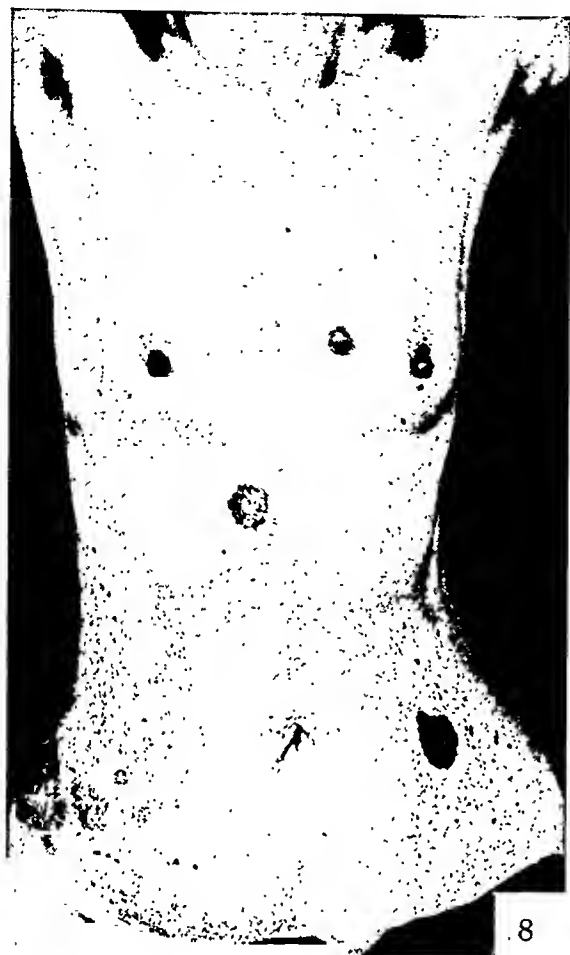
FIGS. 8-10

blasts and their branching twigs creeping between tumor cells, as well as coarse grains of pigment in groups of melanophores in the dermis.

Case 11. #5220.—A medium colored, 40 year old Mahar woman, mother of two children, was admitted for a small circular superficially ulcerating black growth on the face (Fig. 7). She had noticed the growth for nearly two years. It had started as a tiny sore spot, which did not cause any discomfort except that it occasionally irritated her. She accidently injured it two months back and it began to grow rapidly. On examination, a flat black round nodule about 0.5 cm. in diameter was discovered filling the left nasogonial fold. It was slightly raised above the surface (4 mm.) and was not adherent to the subcutaneous structures. There were a couple of spots of surface ulceration. At its periphery the nodule showed a peculiar smooth, translucent appearance which is often noticed in basal cell tumors of the skin.

Microscopic examination of the excised nodule showed a slightly more advanced stage of basal cell carcinoma, dotted with small cystic spaces containing pigment granules and remains of dead tumor cells. The cells inside the tumor cords were elongated and fusiform. Many of them contained a fine dust of brown pigment in their cytoplasm. Golden brownish pigment was also seen in the bodies and branches of the many ramifying cells between the characteristic columnar and fusiform cells. The dendritic cells were easily discernible in unstained sections. There were also groups of melanophores loaded with clumps of pigment in the dermis.

COMMENT

All the tumors belonging to this group were characterized by an insidious onset, and a slow clinical course. Many of them were stated to have originated in a pigmented mole which had been present for a very long time. The tumor had sometimes attracted the attention of the patient after a negligible injury. There was no infiltration of deeper structures nor was there an involvement of regional lymph nodes or distant viscera in any of the cases. There was no preponderance of occurrence in either sex. Their location was not restricted to any particular part of the skin, although the face and particularly the nasogonial

fold appeared favorite sites. The histological findings in these tumors presented several characteristics in common, and the differences were mainly quantitative as regards (a) the size of the lesion and its encroachment on the dermis (b) the relative participation of polygonal prickle cells in the cords of basal cell carcinoma and (c) the amount of pigment visible or demonstrable in the tumor mass. In none of these tumors true nevus-cell accumulations (cell nests, theques) were seen in the dermis and there was nothing to suggest an affiliation of these tumors with the group of benign or malignant melanomas. These tumors appeared to be satisfactorily eradicated by adequate excision or contact radiation therapy.

MULTIPLE BASAL-CELL PIGMENTED TUMORS

Case 12. #14612.—A tall, thin, nervous, wheat-coloured, 51-year old Eurasian married woman was admitted for ulcerated black nodules on the skin. When she was about 30 years old a group of "pigmented moles" reappeared on her body. Some of these had begun to spread into black patches during the last 7 to 8 years. She had developed a "boil" above the pubes which burst and formed a red tumor about 3/4 inches in diameter. It was excised and treated by a surgeon who examined it microscopically and had called it a "rodent ulcer". She was referred for her black patches to a specialist whom she saw after 5 years. The dermatologist found some areas with scabs on the surface. "One old one looked melanotic. This she had for 15 years. The others have been there for 6 years." As he was of opinion that "the appearance of some lesions was like a precancerous condition" and others like that of a "basal cell carcinoma," the patient was referred to the Tata Memorial Hospital. On examination the woman (Figs. 8 and 9) was found to have numerous (over 200) pigmented moles on the neck, the trunk and the thighs. There were no pigmented spots on the face. There were several flat black patches on the trunk which were roughly circular and clearly demarcated from the neighboring healthy skin. The central portions of these patches were superficially ulcerated and were covered with brownish scales of dried secretion. There was also an intensely black nodule on the back, at the waist line

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DESCRIPTION OF FIGURES 8 TO 10

FIGS. 8 and 9.—Case 12.: Front and back views of the trunk showing numerous pigmented moles, two superficially ulcerated areas, and three intense black nodules on the skin.

FIG. 10.—Case 12.: Low power photomicrograph from a section from the flat patch behind the right thigh, showing superficial ulceration and thin branching cords of tumor cells infiltrating into the dermis. Hematoxylin and eosin stain. Mag. × 150.

(2 × 1.5 × 1 cm.) and a flat, superficially ulcerated, slightly pigmented patch on the back of the right thigh (1.5 cm. in diameter). These last two were excised, and a biopsy taken from the edge of the patch on the right loin.

Microscopic examination.—The material obtained after excision was available for study and showed a great variety of structure of basal cell carcinoma type. Curiously enough the flat patch behind the right thigh showed greater anaplasia

15 years, during which time he had developed similar lesions over other parts of the body. The latest of these was about three years old. The ulcer or the lumps did not pain him but he felt an itching sensation over them. On examination a black, firm, flattened mass (3 × 2 × 0.5 cms.) was felt in the skin over the body of the left mandible just in front of its angle. The mass was ovoid, raised above the surface and ulcerated in the center. The surface of the ulcer was covered with

FIG. 11.—Case 13.: Photograph showing the pigmented ulcerating nodule on the left lower jaw.

FIG. 12.—Case 13.: Photograph showing pigmented nodules in the two groins and the rhomboid dry scaly area in the left hypogastric region.

and deeper infiltration by tumor cells (Fig. 10), which were arranged in long thin branching strands, or scattered in small groups of cells in the dermis. The pigmentation was associated with a proliferation of melanoblasts and other changes that have already been described.

Case 13. #3377.—A dark-skinned 76 year old Indian Christian, father of 12 children, was admitted for an ulcerated black nodule over the left lower jaw. He had had a "boil" in the same place when he was 20 years old which had healed after being incised to let the pus out. He developed a small lump (Fig. 11) on the same spot forty years later which had burst and left an ulcer. This ulcer had steadily increased in the course of the previous

dried black scabs. It was freely movable over the subjacent structures. There were three other similar nodules on the trunk. These stood out more prominantly and were less pigmented than the lump on the jaw. They were located over the tip of the left costal cartilage in the right groin and on the left thigh near the attachment of the scrotum. On careful examination, a rhomboid, rough, dry, scaly area on the skin of the left lower abdomen (Fig. 12) was seen that had not attracted the attention of the patient. The area was 4 cm. long at its widest extent and was clearly defined by a black, uneven margin. All these lesions were excised with about 1 cm. of normal skin beyond them and were available for histo-

logical study. A full-thickness skin graft was placed on the raw area left on the face after the excision of the mass. The patient made an uneventful recovery and has not reported since with any recurrence of his disease.

Microscopic examination.—All the lesions including the dry, scaly area showed the structure of a basal cell carcinoma, with slight variations in type in the different nodules. These lesions afforded excellent material for a study of the proliferative changes in melanoblasts, by dopa reaction and silver impregnation. These changes will be referred to later while considering the nature of pigmentation in these tumors.

COMMENT

Multiple tumors of this type are very rare in published reports. The cases reported by Nomland (16), Pautrier and Archambaut (17), and by Nisbet (15) probably belong in this category. In view of the discussion following the case reported by Nomland (19), it is necessary to point out that the areas of predilection for epithelioma adenoides cysticum, *viz.* the lower eyelids, nose and portions of the cheek, were exempt from disease in both the patients described above. Case 13 was a male and there was no evidence of a familial tendency to disease in either case. Further it was found that even though the lesions in both patients were present for a long period the onset of disease was not in early life, nor was an accelerated growth associated with the time of puberty. The similarity between these cases and those reported in the literature consisted in the following features:

1. A clinical resemblance to pigmented nevi without the presence of nevus cells arranged in cell nests (theques), or strands in the dermis.

2. A slow evolution with probable origin in a preexisting pigmented mole. An absence of regional or distant metastases even after many years' existence.

3. Variation in form, color and histological details in different lesions in the same person.

MULTIPLE SQUAMOUS CELL PIGMENTED TUMORS

Case 14. #5608.—A fair-skinned Hindu bania, owner of an electrical appliances shop, was admitted to the hospital on Nov. 16, 1943 with an ulcerated growth at the base of the left palm. He gave the following history about his complaint. A few small dark spots had suddenly appeared at the root of the palm just below the middle of the right wrist 10 years previously. These spots increased slowly in size and had fused to form a firm, dark nodule causing itching and some discomfort. He was treated by his physician with ultraviolet rays which seemed to arrest the growth of the nodule. It started growing again after 2 years. He was treated by "application of radium." The condition improved under this treatment. The lesion became active again after an interval of two years. He was treated with deep x-rays in 1937 and 1940. He denied having had any medicines containing arsenic. He was unable to supply exact information regarding the dosage of the x-ray and radium therapy. On examination a firm ulcerating growth about 5 cm. in diameter was seen in front of the wrist spreading on to the palm of the hand (Fig. 13). The growth was surrounded by a broad zone of depigmented skin and a darkly pigmented ring outside it. The growth was partly fixed to the tendons of the flexor muscles. There was a black rough patch with uneven fissured surface on the right wrist. This was excised and showed closely packed black papillary proliferations of the skin moulded on thin strands of connective tissue. A similar lighter patch was present on the palmar surface of the right middle finger. Both palms showed numerous minute discrete translucent nodules in the skin. A small node was felt in the left axilla. The physical examination revealed no other abnormality except a number of large and small *cafe au lait* spots on the back, chest, abdomen and scalp. The face was free from any blemishes. x-ray examination showed marked decalcification of the bones subjacent to the ulcer but there was no destruction or evidence of involvement of bone structures.

The lesion was treated with deep x-radiation. A total dose of 4000 r units was administered to him during the course of 10 days (85 kv. 1 Al. T. S. D. 15 cm. circular field 6 cm. diameter). The ulcer rapidly healed and the wrist movements improved under treatment. At a follow-up 2 months later it was noticed that he had residual disease and he was given a further total dose of 1500 r units over a period of 1 week (200 kv. 0.5 cu + Al, T. S. D. 50 cms. circular field 5 cms. in diameter). The lesion on the palm became cleaner, but some disease still persisted. One month later the patient appeared with a raised pigmented lesion on the scalp, 2 cm. in diameter which bled easily on touching. This lesion and those on the right hand regressed completely with x-ray therapy, but the lesion on the palm persisted. It was therefore decided to amputate the limb at the middle of the forearm.

The patient returned after six months with a small pigmented papillary lesion on the scrotum and again four months later with similar small lesions on the right thumb and the fingers of the
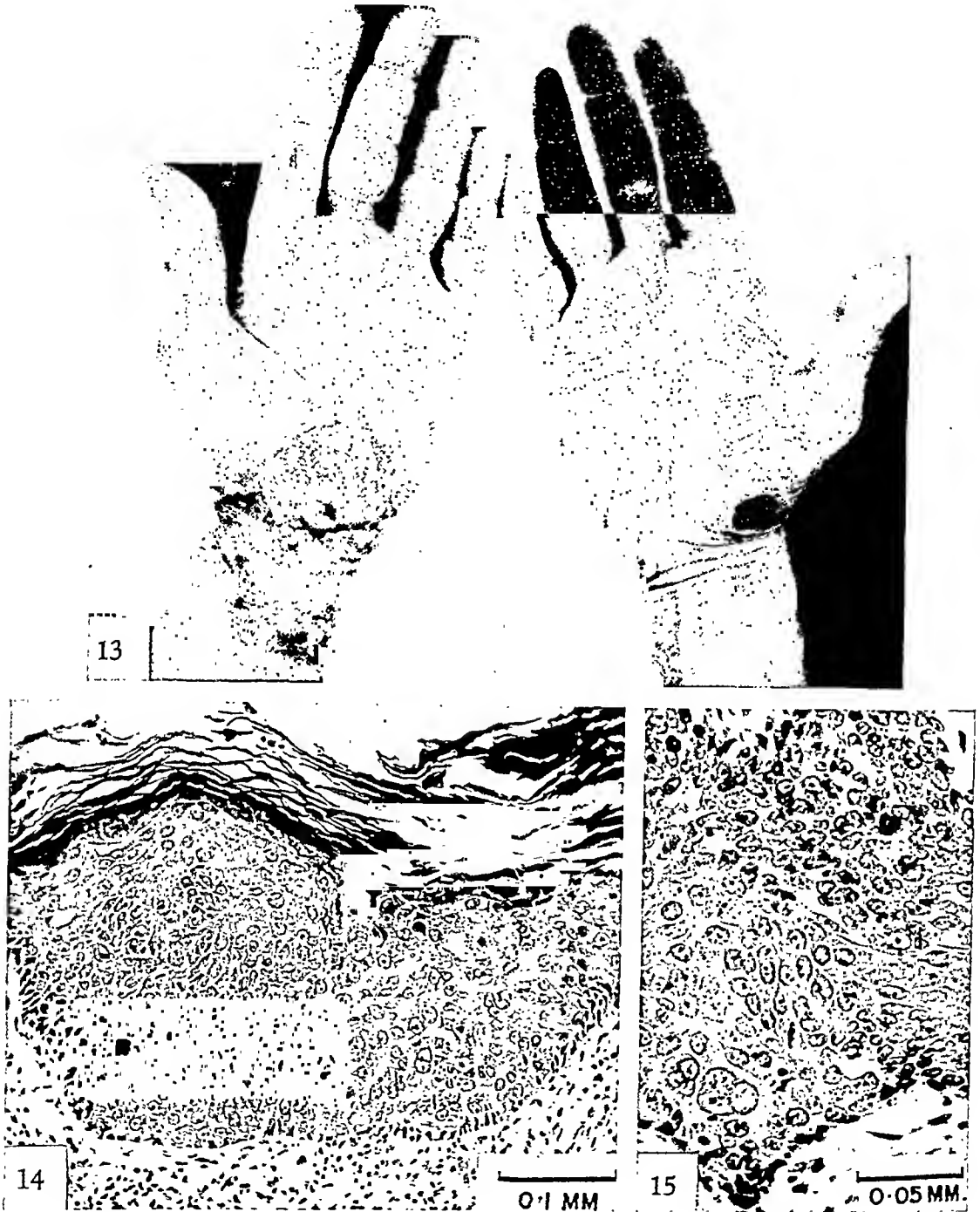
FIG. 13.—Case 14.: Photograph of the palms showing the malignant lesion on the left hand, the darkly pigmented patches on the right, and the minute translucent whitish nodules in the skin covering the palmar surface of the fingers on both hands.

FIG. 14.—Case 14.: High power photomicrograph from one of the minute discrete nodules showing hyperkeratosis, disorganisation of the normal arrangement and stratification of cells, and individual cell keratinisation. Hematoxylin and eosin stain. Mag. × 200.

FIG. 15.—Case 14.: A higher power photomicrograph of a nodule similar to that shown in Fig. 14 presenting the clumping of nuclei, *corps ronds* and monstrous cells. Hematoxylin and eosin stain. Mag. × 375.

right hand. All these lesions rapidly regressed with contact x-ray treatment for 10 days giving a total dose of 4800 r units (50 kv. 1 Al and 4 cm. T. S. D.)

Microscopic examination.—The left hand and the excised lesion from the right hand (b) were available for histological study. The growth on the left palm showed the characteristics of a squamous carcinoma grade III. The pigmented piece excised from the right hand was made up of dermal papillae extended into long branched processes covered with altered stratified squamous epithelium. There was hyperkeratosis, a disorderly arrangement of the cells in the stratified malpighian layer and large monstrous cells with two or three hyperchromatic nuclei. Some cells showed hydropic vacuolation of cells with small pyknotic nuclei. The basal layer of cells was regular, intact, sharply demarcated from the subjacent fibrovascular connective tissue. The small greyish nodules on the palm of the left hand showed the following interesting features.

The surface was covered with several layers of fine lamellae of keratinized material. The epithelium indented the dermis unevenly due to elongation broadening and fusion of rete cones. The minute discrete nodules which were visible to the naked eye were composed of broad epithelial buds pushing into the dermis (Figs. 14 and 15). The cells in the nodule showed a disorganization of the normal arrangement and stratification of cells. There was a wide variation in the size of adjoining cells, with some monstrous cells, and others with several nuclei clumped together, interspersed between normal polygonal cells. Some of the cells showed a characteristic intracellular vacuolation with a preservation of intercellular bridges. In the substance of the neoplastic mass "individual cell keratinisation" (13) and "corps ronds and grains" (7) were seen. These features suggested the change to be Bowenoid in character. It seemed probable that arsenic might have been administered during the course of the varied treatments received by the patient. The skin from the amputated forearm and the hand was therefore analyzed. It gave the following interesting data:

ARSENIC CONTENT OF THE TISSUES

Tissue	Arsenic (as As₂O₃) mgm. per 100 gm.
Healthy skin in Indians*	0.150 (0.125 minimum) (0.175 maximum)
Normal-looking skin from the forearm of the patient†	0.198
Cancer tissue from the wrist†	0.924
Skin containing hyperkeratotic nodules from the palm†	3.036

*From data published by Bagchi and Ganguly (1).
†Technic employed by Maechling and Flinn (10).

Case 15. #2936.—An elderly emaciated Hindu beggar woman, 60 years old, was admitted for a black warty growth on her abdomen. She stated that the growth had started as an intense black spot 7 years earlier and that she was sure that it was not there before that time. The spot had slowly increased in size and had became rough on the surface. Black warty excrescences had slowly grown out of it. She complained of much itching, and occasional bleeding after scratching. On examination, a verrucous mass, roughly lozenge-shaped (17 × 12 cm.) was seen covering most of the left lower abdominal wall (Fig. 16). The edges were serpigenous and stood out clearly from the adjacent normal skin by their deeply pigmented color and elevated contour. The main mass was deep black except towards the middle where it was depigmented and atrophic in places. A second smaller pigmented patch composed of bunches of large and small papillae was discovered in the right loin. The patient did not remember when it had started. The larger growth emitted a faint fetid odor and the warty projections could be pealed off with little trouble and slight bleeding. Clinical examination and skiagraphic studies did not reveal any morbid condition in the gastrointestinal tract. Both lesions were excised along with a narrow margin of normal skin beyond the pigmented border. Skin from the thigh was grafted on the raw excised surface of the bigger lesion. The patient made an uneventful recovery. She has not reported a recurrence of the lesion nor of any fresh outcrops of pigmented spots for the last four years.

Microscopic examination.—(a) The flat heavily pigmented peripheral portion of the tumor mass showed a thickening of the epidermis with broadening, elongation and fusion of rete cones. There was hyperkeratosis and parakeratosis of the epithelial layers. In the thickened epidermis there was a loss of normal stratification of cell layers. Many cells in the malpighian layer were swollen, edematous and vacuolated. The nuclei of several cells were large, hyperchromatic and clumped in groups of three or four. There were numerous dyskeratotic cells with pyknotic nuclei and markedly acidophilic cytoplasm. These cells were always surrounded by a clear space. The basal layer of the stratum germinativum was heavily pigmented. There were numerous diffusely pigmented branching cells scattered between the epidermal cells. In the superficial layers of the dermis there were several melanophores stuffed with pigment, besides numerous clumps of free pigment scattered between connective tissue fibers. The transition between the normal and the affected skin was sharp and sudden.

FIG. 16.—Case 15.: Photograph showing the lozenge-shaped verrucous pigmented mass on the left lower abdomen.

FIG. 17.—Case 15.: Low power photomicrograph showing cords and strands of proliferated epithelial cells with central areas of keratinisation infiltrating the subjacent tissue. Hematoxylin and eosin stain. Mag. × 80.
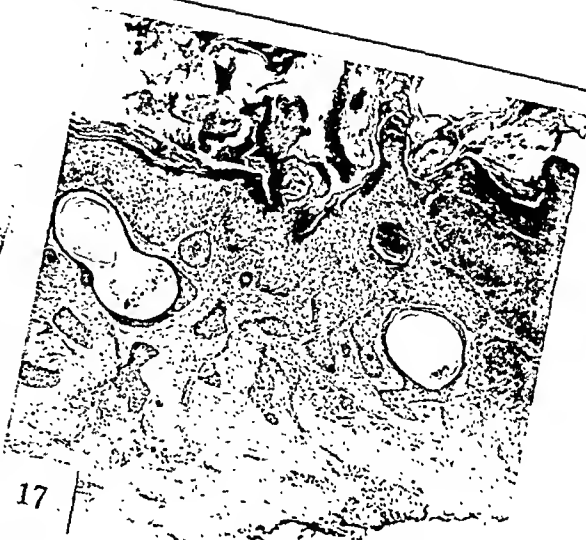
FIG. 18.—Case 15.: A higher power photomicrograph showing the thickened walls of a sweat gland duct and the adjacent epithelium presenting the characters of an intra-epithelial carcinoma. Hematoxylin and eosin stain. Mag. × 200.

(b) The intermediate zone showed several branched filamentous epidermal processes which were covered with several layers of closely applied keratinized lamellae. The epithelium was thickened by an increase in the cell layers and a more pronounced Bowenoid alteration in the character of cells. The peripheral processes were deeply pigmented whereas the more centrally placed filaments of epithelial cells were completely devoid of pigment.

(c) The central portion of the lesion shows a relative thinning of the epidermis accompanied by an invasive proliferation of epithelial cells (Fig. 17) into the subjacent dermis. The proliferating

cells were arranged as cords or strands in the dermis with the development of characteristic epithelial pearls. In some areas the cells of the stratum malpighi showed hyperplastic prickle cells without any of the Bowenoid changes described above. There was a well developed stroma reaction in the dermis mainly consisting of lymphocytes, with few histiocytes and newly formed blood capillaries. The remarkable feature of this area was a complete lack of pigment in epithelial cells and an absence of the branching melanoblasts. There were no melanophores or free pigment clumps in the dermis.

The ducts of the sweat glands (Fig. 18) and the hair follicles in all these sections were involved in the neoplastic process without being altered. The lining of some ducts in the central area however showed the characters of an intra-epithelial carcinoma. The elastic tissue was pushed deeper in all these sections by the inflammatory exudate and the newly formed connective tissue stroma underlying the altered epithelium. The dopa reagent evinced an intense positive reaction in the proliferated and migrating dendritic cells in the peripheral pigmented regions of the tumor mass.

The histological study of the tumor tissue suggests that it belongs to a type of Bowenoid dermatosis, beginning as a deeply pigmented patch which becomes depigmented in the central older area. The depigmented area is characterized by the development of a slowly invading carcinoma of the prickle-cell variety.

Comment

The last two cases show the development of a Bowenoid change in the epidermis antecedent to an invasive cell proliferation and the formation of a typical squamous-cell carcinoma. The interesting feature of these cases is the melanotic pigmentation of the tumor tissue. Bloch (3) had suggested that in the basal cell tumor described by him the pigmentation was the essential feature and that the rest of the structure was secondary. Subsequent pigmentation in patches of Bowen's disease of the skin has often been described, but in both the patients referred to here, the lesions started as black patches and the growing peripheral areas of the fully developed tumor were deeply pigmented as a result of the proliferation and activity of dendritic melanoblasts. This activity and proliferation did not keep pace with the neoplastic growth of epithelial cells. The older, central, fully developed portions of the tumor therefore remained unprovided with pigment-elaborating cells, and became colorless.

General Considerations

I. Deposition of Pigment in Tumors of the Epidermis

(a) *Nature of the pigment.*—The pigment gives the usual chemical reactions of melanin. It is insoluble in all the ordinary solvents except strong solutions of alkalies, in which it dissolves with some difficulty. It could be slowly bleached by the action of strong sunlight. It does not give the Prussian blue reaction for iron, but is easily impregnated with silver solutions.

(b) *Distribution of the pigment in tumor tissue.*— A naked-eye examination of unstained sections shows that the pigment is unevenly distributed in lobes of tumor tissue (Fig. 4). It is more densely deposited in the peripheral cords and is scantier in the central areas. It occurs as, (I) a fine dust of golden or dark brown particles in the cytoplasm of cells in stratum germinativum and some tumor cells; (II) as diffuse homogenous brown coloring material in the cell body and dendrites of degenerating melanoblasts; (III) as coarse dark brown grains in the dermis or as clumps in the bodies of melanophores, and (IV) as large amorphous masses in cystic spaces in the center of tumor lobules. In the cells of the stratum germinativum it tends to be more closely aggregated in the zone immediately outside the nuclear membrane.

(c) *Pigment-forming cells (Melanoblasts).*—The most interesting feature of these tumors is a proliferation of dendritic cells. The change in the epithelial cells, as one approaches the tumor mass, is accompanied by an alteration in appearance and an increase in number of the dendritic cells (Fig. 19). In this zone the melanoblasts become more numerous and send out a rich brush of long thin processes between the epidermal cells, while still retaining their place in the basal layer of the stratum germinativum abutting against the dermis. These dendritic cells are not easily recognizable by the usual staining methods. They are, however, clearly depicted owing to the presence of a dopa-oxidase in their cell cytoplasm and the fine protoplasmic ramifications, and by their ability to reduce silver salts from solutions. As the expanding epithelial cones fuse and assume the shape of a growing bud the proliferated dendritic cells form a rich protoplasmic network on its dermal surface (Figs. 20 and 21). The dendritic cells move away from the periphery as the epithelial bud grows in mass. They however retain contact with the dermis by a thick process which usually ends in a globular or mushroomed terminal (Figs. 22 and 23). The dendritic cells

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20

0·1 MM.

0·05 MM.

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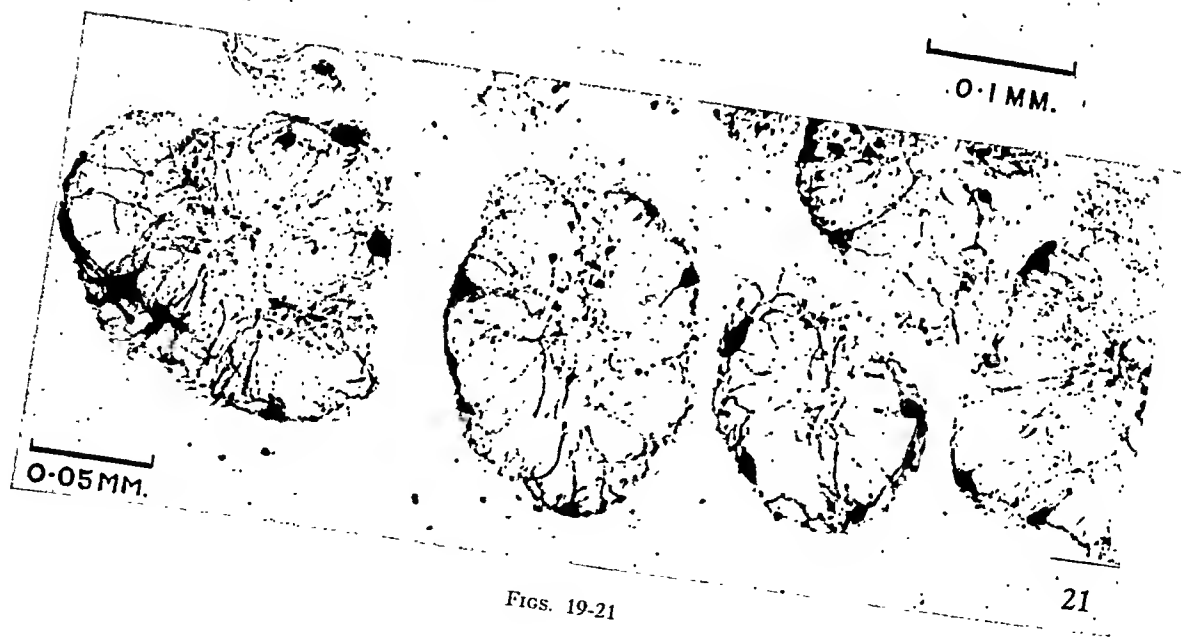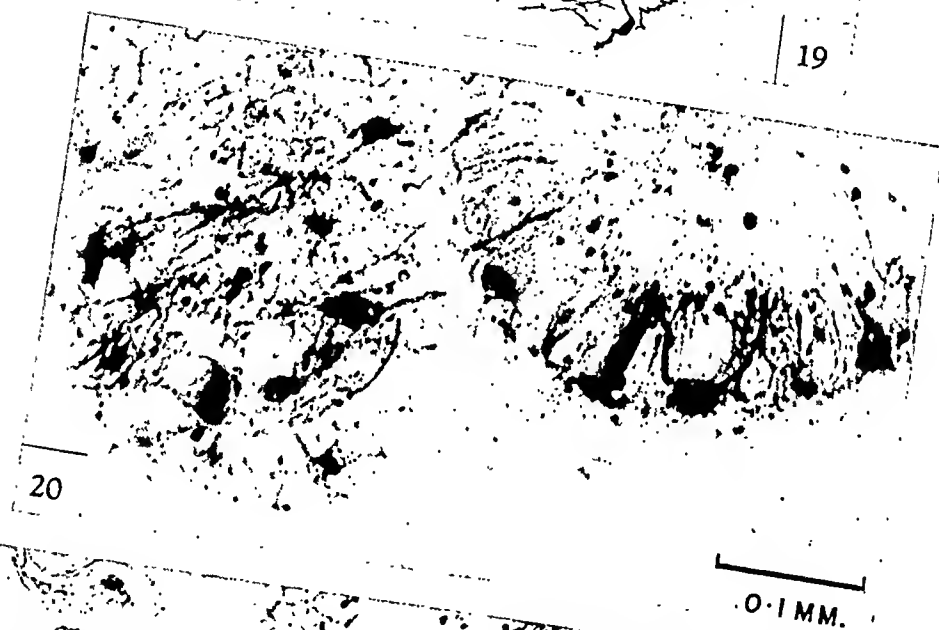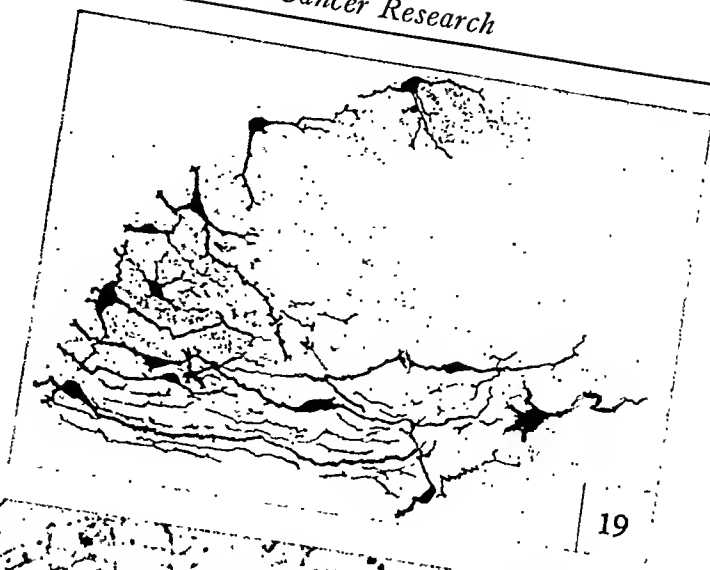
Figs. 19-21

assume an elongated spindle shape on receding from the basal layer. The dendrites become fewer, longer and more filiform as the cells migrate away from the periphery and are caught up in the mass of neoplastic epithelial cells. This migration of dendritic cells has been elegantly described by Masson (11) and by Caudiére (5). These cells appear to be unable to transfer the precursor of the pigment to the adjacent tumor cells, or alternatively the tumor cells lose the capacity of accepting and elaborating the pigment. The pigment, therefore, begins to accumulate in the cell body and branches of the dendritic cells. After their being bereft of their dermal associations, the dendrites thicken and coarsen. Gradually the cells become shrunken and are shorn of most of their branches (Fig. 24). A thick short stump may remain attached to the shrunken, degenerating cells, before they are sloughed off into the amorphous debris. Similar degenerative changes in melanoblasts have been described by Schneider (20) in luetic infiltrations of the epidermis with *Trep. pallidum* and observed by us in a case of a fungus granuloma of the nipple in a 55 year old male.

It is necessary, however, to determine whether the evident increase in the number of dendritic cells at the growing edges of these tumors is genuine or spurious. Rous (18) and Beard (2) have observed that Shope papilloma in rabbits is frequently grey, brown or black with melanin. They noticed (2) that pigmented tumors developed "only where the hair was pigmented ... When the first epithelial thickening took place, melanoblasts similar to those nearby the unaffected epidermis proliferated in the basal part of the papillomatous epidermis and often became extraordinarily abundant, and black with pigment." They were of the opinion that "pigmented growths arise because these cells (melanoblasts) become involved in the pathological process though not themselves affected by the virus." Masson (12) while discussing the appearances in macular pigmented nevi has expressed the following view. "At first it was attempted to show this excess of branching cells to be the result of their hyperplasia. I do not believe this to be true." He attributes the apparent increase in number of these cells "to an exaggeration

of amboceptric differentiation, to the detriment of malpighian differentiation." Caudiére has also expressed the opinion that No part of them [pigmentary cells] shows signs of proliferation. The tumors that do present these characteristics are symbiotic, pigmentary cell epitheliomas." (5). These views deserve most careful consideration, although it must be admitted that the appearances observed in the tumors described above are very suggestive of a true hyperplasia in the spreading zone of the tumor tissue. It is also difficult to accept the opinion of Caudiére that "They are not pigmentary tumors, they are pigmented tumors" (5), as in several cases the lesions start as a deeply pigmented patch, which may later grow discolored towards the central part of the lesion. The pigment-producing cells are actively associated with the growth of neoplastic tissue, although they fail to keep pace with the increase in number of other epithelial cells and are later completely choked by them.

(d) *Accumulation of pigment in tumor tissue.*—The dark color of these tumors is due not only to the presence of pigment particles in the melanoblasts and some tumor cells, but also to a lack of normal elimination of dead epithelial cells. Towards the center of the tumor lobes the keratinized bodies of epithelial cells and the degenerated bodies of melanoblasts are cast off in the debris of necrotic material. The pigment, however, remains unaltered and is retained in cystic spaces and fissures or in the widened follicles involved in the neoplastic process. There appear also groups or circumscribed masses of bulky ovoid or fusiform cells in the superficial layers of the dermis heavily loaded with coarse granules of brown pigment (Fig. 25). They often lie in close proximity to the lobes and cords of tumor tissue and are evidently macrophages which have engorged themselves with pigment. The exact source of pigment in these cells is not very clear. These macrophage cells are never encountered in the body of the neoplastic cords. They are only seen in the connective tissue stroma separating the lobes. These appearances suggest that the macrophages take up formed pigment which is "spilled over" in the dermis and which is not retained by tumor cells. These macrophages have, therefore, been correctly termed melanophores and are distinct in origin

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DESCRIPTION OF FIGURES 19 TO 21

Fig. 19.—A composite picture of camera lucida drawings of seven fields, from a biopsy of tissue in case 13. It shows an increase in the number and the alteration in the morphological characters of the melanoblasts in the peripheral zone of the tumor mass. Dopa reaction.

Figs. 20 and 21.—A rich protoplasmic network of the processes of dendritic cells on the surface of epithelial buds from case 13. Dopa reaction. Fig. 20, Mag. $\times$ 200; Fig. 21, Mag. $\times$ 375.

and evolution from the melanoblasts described above or the nevus cells encountered in benign or malignant melanomas

II. Regression of Neoplastic Changes

The central area of the lesion in Case 8 showed a tendency towards healing and a replacement of the neoplastic cells by epithelium without evident proliferative activity. Similarly in several centrally located areas in Case 15 there was a disappearance of the Bowenoid change and its replacement by a normal looking stratified squamous epithelium. Such retrogression of experimental tar cancer has been reported. Rous and Kidd (18) observed a raised ulcerated disc after 5 months tarring in one of their rabbits. The growth took on an invasive character during the next 4 months. Later it began to grow smaller and disappeared completely in another 4 weeks. A similar carcinoma with metastases was described by Yamagiwa and Ichikawa, which retrogressed after 630 days of growth. The suggestion therefore that proliferating epithelial buds or Bowenoid changes should be interpreted as a carcinoma could not be accepted without reservations. These conditions should be looked upon as precancerous changes which have the potentiality of developing into a carcinoma with the introduction of other factors which are not so well understood at present.

III. Development of Cancer in the Altered Epidermis

The invasive character of cell proliferation in the central portions of the lesions in Cases 11 and 15 reemphasize the importance of a distinction between tumor inception and tumor formation. The latter condition ensues only when appropriate conditions exist in a area for an infiltrative growth of tumor tissue. This question has been fully discussed previously (9) and need not be entered into again. The importance of a precocious stroma reaction some distance away from the proliferating epithelial buds has been stressed as an influential factor in the limitation of invasive characters in some of the skin cancers by Masson (12) (*stroma reaction précoce*) and it is likely that only when

this character fails in the altered dermis, a true carcinoma results.

IV. Spread of Tumors

The spread of these tumors presents certain interesting features. In basal cell carcinomas one is struck with multiple microscopic foci of epithelial proliferation, and the spread along the surface and in depth of the lesion is to a certain extent due to a fusion of these foci and to a growth in mass which results from continued cell division. In the case of the Bowenoid changes in the skin there are two possible methods of lateral spread: (I) an intra-epidermal migration of neoplastic cells as in Paget's disease of the nipple [Muir (14)], or (II) a progressive Bowenoid transformation of normal epithelium in response to an inducing agent continuously being elaborated by active or degenerating neoplastic cells. The latter possibility appears more likely in view of the fact that the line of demarcation between the normal and altered skin is usually very sharp and also because of the arrest of disease after surgical extirpation of affected area of skin. A careful study of the epidermis at the border zone shows few swollen prickle cells just above the normal cells of the basal layer, but no frank neoplastic cells could be detected between normal cells of the malphigian layer as in Paget's disease of the breast. The material at our disposal is inadequate for a solution of this problem, and its elucidation would probably follow the experimental studies now in progress under Cowdry at St. Louis (6).

SUMMARY

1. Fifteen cases of pigmented lesions of the skin which were not melanomas have been reported. Four cases of single pigmented epitheliomas of the skin and four others with multiple lesions have been described.

2. The necessity of a histological diagnosis in all these cases has been emphasized and their relatively benign course has been stressed.

3. The biological nature of these lesions and the role of melanoblasts in their evolution has been discussed.

DESCRIPTION OF FIGURES 22 TO 25

Figs. 22 and 23.—Case 13.: Photomicrographs showing the migration of melanoblasts and the thick processes abutting against the dermis. Dopa reaction. Fig. 22, Mag. × 375; Fig. 23, Mag. × 80.

Fig. 24.—Case 12.: Photomicrograph showing the degenerative changes in the migrated melanoblasts, with a shrinkage of cells and loss of dendrites. Silver impregnation. Mag. × 80.

Fig. 25.—Case 13.: A higher power photomicrograph showing the three types of accumulation of pigment in the growing tumor buds, (1) in the debris of necrotic material at the center of tumor lobes (2) in the bodies and process of melanoblasts (3) in groups of melanophores in the dermis. Dopa reaction. Mag. × 150.
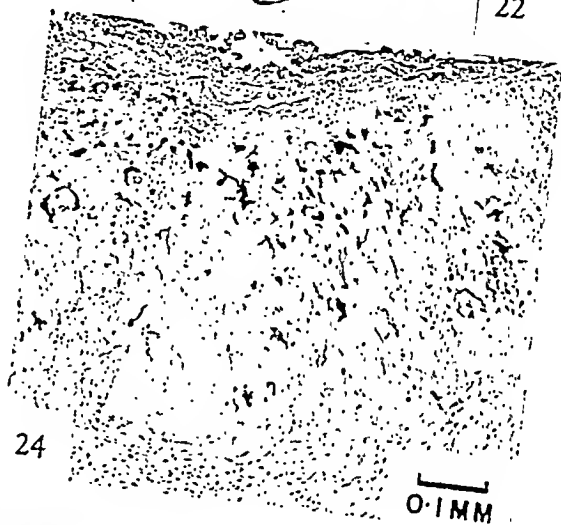
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22

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23

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Figs. 22-25

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HORMONAL IMBALANCES AND TUMORS OF ENDOCRINE GLANDS. W. U. GARDNER. (Department of Anatomy, Yale University School of Medicine, New Haven, Conn.)

Tumors of interstitial cells of the testes, pituitary glands, ovaries and adrenal glands have been induced experimentally in animals under conditions of hormonal imbalance produced by the addition of sex hormones, the removal of the sources of intrinsic sex hormones, or the production of excessive gonadotrophins. In most cases genetic factors are of importance in that the tumors are strain-limited. Testicular interstitial cell tumors appear in estrogen-treated mice of the A and JK strains and in their first generation hybrids. The pituitary is assumed to be involved in formation of these tumors. Chromophobe hyperplasias and adenomas occur in estrogen-treated mice of the C57 strain and in their hybrids but rarely in mice of other strains or hybrid groups. The simultaneous administration of androgen partially inhibits their appearance. Whether hormonal mechanisms prevent their appearance in the mice of the resistant strains is not known. Ovarian tumors occur in intrasplenic transplants of ovaries. Under such conditions the ovaries are exposed to excessive intrinsic gonadotrophin, presumably follicle-stimulating hormone, although sex differences exist. The ovarian tumors appearing in roentgen irradiated mice may be explained on a humoral imbalance basis. Adrenal cortical tumors also appear in mice (Woolley) subsequent to gonadectomy at birth or even when older (Gardner). These tumors as well as the testicular interstitial cell and ovarian tumors mentioned above produce physiologically active substances. These tumors will be discussed from some of their genetic and hormonal interrelationships.

COMPARISON OF THE CARCINOGENIC ACTIVITY IN EXTRACTS OF HUMAN LIVER AND OTHER HUMAN AND ANIMAL ORGANS. PAUL E. STEINER, D. WARREN STANGER, and MIRIAM BOLYARD. (Department of Pathology, University of Chicago, Chicago, Ill.)

Ethylene dichloride extracts after saponification were prepared from pooled human livers, kidneys, spleens, hearts and colons. The extracts were made in duplicate from cancer-bearing and noncancer-bearing patients. Similar extracts were made from pooled livers of stillborn infants, swine livers, bovine livers, and swine hearts. The extracts were tested for carcinogenic activity by subcutaneous injection into 1,044 mice of C57 black, A,

or our albino strains. The percentage yield of sarcomas at the site of injection in C57 black mice surviving for 6 months was: Noncancerous livers, 58.7; cancerous livers, 14.8; cancerous spleens, 10.4; livers of stillborn infants, 8.1; swine livers tested in strain A mice, 7.5. The other extracts were essentially noncarcinogenic.

THE LOCALIZATION OF STEROIDS IN NORMAL AND CANCEROUS TISSUES BY THE USE OF RADIOACTIVE ISOTOPES AND HISTOCHEMICAL METHODS. S. ALBERT, J. COHEN, R. D. H. HEARD, and C. P. LeBLOND. (Departments of Anatomy and Biochemistry, McGill University, Montreal, Canada.)

By using fuchsin-sulphurous-acid and 2,4-dinitrophenylhydrazine, two reagents supposedly specific for ketosteroids, it can be shown that the histochemical reactions thus obtained in tissues are not suppressed by the removal of ketosteroid-producing organs. In normal and cancerous animals the most intense reactions are found in the ovary, testis, adrenal and accessory sex organs, with little or no reaction in cancerous tissue. It is concluded that these reactions reveal the presence of a non-ketosteroid substance, probably an acetal phosphatide of the plasmalogen family, which may be linked with steroid metabolism.

Using α-estradiol iodinated with radioactive iodine, it was possible to follow the distribution of this compound in the tissues of cancerous mice by means of the Geiger counter. It was found that after 10 hours the largest concentration of this compound occurred in the gastrointestinal tract, feces and urine, while only minute amounts occurred in the genital organs, accessory sex organs and cancerous tissues.

A PHYSIOLOGICAL MEASURE OF HOST-TUMOR RELATIONSHIP AS SHOWN BY A TRANSPLANTABLE MOUSE RETICULOENDOTHELIOMA. ARTHUR M. CLOUDMAN. (Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

Measurable physiological changes have been produced by parabiotic operations in mice normally refractory to the transplantable reticuloendothelioma, C198. This leaden strain tumor always involves the liver of susceptible leaden mice that receive subcutaneous implants of tumor tissue. All other pure strains of mice are resistant to this tumor when the usual technics are employed in tumor tissue transfer.

The transfer of some substance or substances through the medium of body tissues and fluids not normally introduced frequently make refractory C57 black strain mice serve as a successful host for tumor C198. After the altered host has progressively grown the tumor for a certain time interval and the mass has become sizeable the tumor itself undergoes a physiological change. After this the tumor can be easily transferred to members of the C57 black strain. However, it will still grow in the leaden mice. Furthermore, whatever tumor change was induced by growth in a black mouse is weakened or lost by growth for one tissue transfer generation in a leaden mouse.

The data presented reveal that (a) parabiosis alters refractory C57 black strain mice, making many of them susceptible to implants of tumor C198; (b) the altered host can change the implanted tumor; (c) this changed tumor can be successfully transferred to other C57 black mice; and (d) C57 black mice growing the altered C198 tumor remain resistant to unaltered C198 taken directly from a leaden donor mouse.

THE NEOPLASTIC TRANSFORMATION OF GRANULOSA CELLS IN GRAFTS OF NORMAL OVARIES INTO SPLEENS OF GONADECTOMIZED MICE. J. FURTH, and H. SOBEL. (Department of Pathology, Cornell University Medical College, New York, N. Y.)

Growth of granulosa cells were produced in 29 (67 per cent) of 43 mice by grafting fragments of normal ovaries into the spleens of gonadectomized mice as described by Biskind and Biskind. After intrasplenic subpassages into gonadectomized mice, the splenic growth in one of these mice became transformed into a neoplasm readily transplantable into the subcutaneous tissue of normal mice and occasionally metastasizing to the lung (Strain B1). A second transplantable strain (B2) was derived from another mouse that had a splenic growth of granulosa cells with secondary nodules in the liver. This growth proved readily transplantable in the spleen, from which it frequently metastasized to the liver, but not in the subcutaneous tissue. The secondary changes in mice bearing these 2 transplantable tumors indicate the discharge of estrogens by the tumor cells. The blood volume of mice bearing subcutaneous tumors of these strains is elevated and their livers show cavernous congestion characteristic of hypervolemia. These experiments serve to illustrate how hyperplasia of normal cells can lead to neoplasia and enable an analysis of the factors bringing about this transformation.

FURTHER STUDIES ON THE PATHOGENESIS OF THE OVARIAN TUMORS IN MICE. M. H. LI, and W. U. GARDNER. (Department of Anatomy, Yale University School of Medicine, New Haven, Conn.)

Our previous experiments demonstrated that a pituitary-gonadal endocrine imbalance may be induced by the intrasplenic transplantation of ovaries in castrated male and female mice and that the imbalances may result in the formation of granulosa cell tumors, luteomas, or mixed cell tumors.

Most extensive studies have been made by using inbred mice of the A, C3H, and C57 strains, and several groups of hybrid mice (A × C3H and CBA × C57). Ovarian tumors have appeared in intrasplenic transplants in mice of the A, C3H and C57 strains and in hybrids; there is apparently no strain limitation in the development of ovarian tumors in intrasplenic autotransplants or homotransplants of ovaries in castrated mice. Ovarian tumors have not been observed in the intrasplenic ovarian transplants in unilaterally gonadectomized male and female mice. The formation of tumors in the intrasplenic ovarian transplants was prevented by weekly administration of small doses of α-estradiol benzoate or testosterone propionate. Similar treatment of progesterone, however was not effective. Daily injection of a gonadotrophic hormone from pregnant mare serum for short periods exerts a stimulating effect on the growth of the transplants and on tumor formation. These observations are interpreted to substantiate further the assumption that overaction of gonadotrophic hormones is responsible for the genesis of the ovarian tumors in mice.

CORRELATION OF A BIOLOGICAL TEST WITH CLINICAL DIAGNOSIS IN HUMAN MALIGNANCY. HOWARD H. BEARD, SAMUEL L LIBERT, and B. HALPERIN (by invitation) (Department of Physiological Chemistry, The Chicago Medical School, Chicago, Ill.)

Forty known malignant urines were extracted with an equal volume of alcohol and ether for 2 days in the small Koch extractor. The process was then repeated for another 2 days. Solvents were combined and evaporated under reduced pressure and the water residue diluted so that 2 cc. represented 100 cc. of the original urine. This amount was injected intraperitoneally into immature white rats and the animals were sacrificed from 1 to 4 days later. Litter mates of the same sex and approximate body weight were used as controls without injection The gonads, spleen and body weight were made soon after death by an overdose of nembutal. The body weight/gonad and body weight/spleen ratios were then calculated for all the animals. In 39 of the 40 known malignant urines these ratios decreased from 20 to 80 per cent and this observation constituted the biological test of malignancy. Nonmalignant urines and those from normal individuals gave ratios that decreased from the control ratios by less than 15 per cent and were considered negative. The average degree of hypertrophy observed was as follows: spleen, 483 to 673 mgm. (39 per cent); male gonads, 1283 to 1991 mgm. (55 per cent), and female gonads, 219 to 365 mgm. (72 per cent). Histological studies showed an intense passive hyperemia of the spleen and intense spermatogenesis in the testes. The female gonads were not sectioned. These results are in agreement with those of Roffo, and Krebs and Gurchot. It is concluded that *all* malignant urines so far tested contain a cancer hormone (probably of sterol nature) which is the cause of the biological test described above. We believe

that this hormone acts through the pituitary to produce increased amounts of a gonadotrophic hormone which is the immediate cause of the hypertrophy of the spleen and gonads. We are not yet convinced that this is an Ascheim-Zondek test but further work may prove this to be so.

THE EXCRETION IN THE URINE OF METABOLITES OF ADRENAL CORTICAL HORMONES IN HEALTH AND DISEASE, INCLUDING NEOPLASTIC GROWTH. KONRAD DOBRINER, S. LIEBERMAN, and C. P. RHOADS. (Sloan-Kettering Institute for Cancer Research, New York, N. Y.)

Two metabolites of adrenal cortical hormones, androstanediol-3α, 11β-one-17 (Mason and Kepler: *J. Biol. Chem.*, 161:235. 1945) and etiocholanol-3α-dione-11, 17 (Lieberman and Dobriner: *J. Biol. Chem.*, 166:773. 1946), have already been isolated from the urine of normal and diseased persons. The purpose of the present communication is to report the isolation from urine of another steroid of adrenal cortical origin, Δ 9-11 etiocholenol-3α-one-17, which is probably a dehydration product of etiocholandiol-3α, 11β-one-17.

Attention is called to the fact that this newly isolated compound has been found quite commonly, although not universally, in the urine of patients with disease states: cancer, lymphatic leukemia, hypertension and adrenal cortical disorders of the Cushing's syndrome type, but has been found in the urine of only 2 of 23 normal subjects. It is suggested that this compound is a product of deranged metabolism of adrenal cortical hormones or a metabolite of an abnormal precursor.

EFFECT OF DIET DEFICIENT IN CERTAIN AMINO ACIDS ON THE INDUCTION OF LEUKEMIA IN dba MICE. JULIUS WHITE, FLORENCE R. WHITE, and G. B. MIDER. (National Cancer Institute, Bethesda, Md., and Department of Surgery, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

A comparative study was made of the restriction of cystine, lysine and tryptophane, respectively, on methylcholanthrene-induced leukemia in strain dba mice. Each of the diets employed was so restricted in one of the foregoing amino acids that growth of young mice was prohibited, but indefinite maintenance was possible. The same diets, each supplemented by the amino acid in which it was deficient, permitted good growth. There was no significant decrease in the incidence of leukemia among the mice on diets restricted in either lysine or tryptophane. There was a reduction in the incidence of leukemia from 92.1 per cent for the control group to 55 per cent in the group of mice whose diet was restricted in cystine. The data indicate that under the conditions of the experiment, cystine played a role in the development of leukemia not associated with its properties as an essential amino acid for growth but some other attribute not yet determined.

INFLUENCE OF ESTROGENS AND ANDROGENS ON DEVELOPMENT OF DIETARY CIRRHOSIS IN RATS. IRA T. NATHANSON and PAUL C. ZAMECNIK. (Medical Laboratories, Collis P. Huntington Memorial Hospital, Harvard University; and Tumor Clinic, Massachusetts General Hospital, Boston, Mass.)

Ninety-nine 6 week old female and forty-eight 8 month old male Sprague-Dawley rats were divided into groups as follows: (1) control diet, (2) control diet plus androgen, (3) control diet plus estrogen, (4) modified Gyorgy diet, (5) modified Gyorgy diet plus androgen and (6) modified Gyorgy diet plus estrogen. 2.5 mgm. of testosterone propionate and 0.2 mgm. of estradiol dipropionate were injected subcutaneously into the appropriate groups twice a week for 4 months. Animals from all groups were sacrificed at intervals, and the experiment terminated at 8 months. There was marked fatty infiltration in groups 4 and 5. Group 6 animals, however, showed little fatty infiltration, particularly in the male rats, indicating a striking lipotropic effect of estrogen.

The series of the female rats on the experimental diet showed both gross and microscopic evidence of cirrhosis. Androgens appeared to aggravate the cirrhosis, while estrogens appeared to have an ameliorating effect.

EFFECT OF VARYING THE PROTEIN (CASEIN) CONTENT OF THE DIET ON THE FORMATION OF TUMORS IN THE MOUSE. ALBERT TANNENBAUM and HERBERT SILVERSTONE. (Department of Cancer Research, Michael Reese Hospital, Chicago, Ill.)

Since it is generally believed that protein metabolism may play an important role in the formation of tumors, the effect of different levels of dietary protein (casein) was studied. "Synthetic" diets were utilized and protein levels of 9, 18, 27, 36 and 45 per cent were obtained by substituting casein for cornstarch. All other components of the diets were left unchanged. The modifying effect of the level of protein was evaluated with the carcinogen-induced skin tumor, the spontaneous mammary carcinoma, and the spontaneous hepatoma of the mouse.

No significant effect on either the incidence of induced skin tumors or their average time of appearance was observed. With the spontaneous mammary carcinoma, no difference in incidence was found but the tumors may have appeared somewhat earlier, on the average, in the group being fed 18 per cent casein. In the three groups of C3H male mice receiving diets containing 9, 18, and 45 per cent casein, the percentage of spontaneous hepatomas at 13 months were 11, 61, and 38 respectively, indicating that the "low" and "high" protein diets led to fewer tumors than the diet with "moderate" protein.

It may be concluded that varying the protein (casein) content of the diets, within the limits as indicated, probably has little effect on the formation of many types of tumors, but may have a significant effect on certain special kinds.

DIFFERENCE IN ACTIVATION OF PROTEO-LYTIC ENZYMES IN NORMAL LIVER AND HEPATOMA, AS DETERMINED BY MEANS OF A NEW MONOMETRIC METHOD FOR FOLLOWING PEPTIDE CLEAVAGES. PAUL C. ZAMECNIK and MARY L. STEPHENSON. (Medical Laboratories, Collis P. Huntington Memorial Hospital; and Tumor Clinic, Massachusetts General Hospital, Boston, Mass.)

A manometric method has been devised, which facilitates the study of reaction kinetics involved in the hydrolysis of tyrosine-containing peptides by catheptic enzymes. This method depends on the inclusion in the reaction mixture of a bacterial decarboxylase, which liberates carbon dioxide from *l*-tyrosine as the latter is split from peptide linkage. Since the decarboxylase is present in excess, the rate of carbon dioxide production from tyrosine reflects the rate of the peptide cleavage. This method makes it possible to follow in detail the activation mechanism of the catheptic enzyme.

Ultrafiltrates have been prepared from normal rat livers, and from primary hepatomas induced by butter yellow. The ultrafiltrates of normal livers and of the non-malignant portion of the hepatoma-containing livers activate a purified catheptic enzyme more than ultrafiltrates prepared from hepatoma nodules.

THE INHIBITING ACTION OF AMORPHOUS AND CRYSTALLINE PENICILLIN AND STREP-TOMYCIN PREPARATIONS ON THE META-BOLISM OF TUMORS AND OTHER TISSUES. DEAN BURK, MARIE L. HESSELBACH, and CLARA E. FISCHER. (National Cancer Institute, Bethesda, Md.)

Amorphous preparations of penicillin have been found to produce a marked inhibition of respiration of tumors and normal tissues (*e.g.*, spontaneous breast adenocarcinoma, transplanted Barrett C3HBA adenocarcinoma, Earle L sarcoma, kidney, spleen, and liver of mice). The inhibition is immediate (detectable manometrically within a few minutes) and progressive, attaining practical completion (95 to 100 per cent) within one to several hours, depending upon the amorphous preparation and concentration employed (range, 0.1 to 10 mgm./cc.). Penicillin G several-times recrystallized (1,660 Oxford units/mgm.) was approximately one-tenth as inhibitory on a weight basis as several amorphous preparations assaying 1,000 to 1,500 Oxford penicillin units/mgm. Whether this small activity is due to the crystalline penicillin itself, or to possible traces of the "amorphous factor" still extant as impurity, remains to be determined. Crystalline streptomycin salt was still less inhibitory on a weight basis.

Treatment of various amorphous preparations with *B. subtilis* penicillinase, to remove essentially all penicillin activity against microorganisms, reduced the respiration inhibiting activity per mgm. by 25 to 75 per cent, depending upon the ratio of the amorphous factor to penicillin in the preparation. The amorphous factor can thus act on respiration independently of the presence of penicillin. Synergistic action (as occurs in the case of the

enhancement factor of Welch, Randall, and Price) has not been definitely indicated. In any event, metabolic analysis offers a rapid and comparatively sensitive method of assay. Tumor glycolysis was nearly as subject to inhibition by the amorphous factor as was respiration.

EFFECTS OF AN ASCORBIC ACID DEFICIENCY ON TUMORS. WILLIAM v. B. ROBERTSON, A. J. DALTON, and WALTER HESTON. (National Cancer Institute, Bethesda, Md.)

The effect of an ascorbic acid (vitamin C) deficiency on tumors was studied on transplants of a fibrosarcoma (N.C.I. − C − 2663) in an inbred family of guinea pigs. Animals were maintained on an adequate diet until the tumor transplants became palpable, and then were placed on the scorbutigenic diet.

After the guinea pigs had been fed the vitamin C-free diet for 2 weeks, the tumors appeared to become attached to the skin and belly wall, whereas transplants in animals on an adequate diet were loose and easily movable. At necropsy, the scorbutic guinea pigs were found to have large amounts of hemorrhagic connective tissue connecting the tumor capsule with the deeper layers of epidermis and with the musculature of the body wall.

Transplants of this fibrosarcoma show a core of central necrosis surrounded by a margin of healthy tumor tissue, but the tumors in scorbutic hosts showed not only much larger areas of central necrosis but also many areas of focal necrosis scattered throughout the periphery.

The ascorbic acid concentration of the tumors in the scorbutic cavies was essentially zero; that of the non-neoplastic tissues, although considerably below normal, was still appreciable.

The collagen concentration of tumors in the scorbutic animals averaged 3.7 per cent, as compared with the concentration of 8.9 per cent found in the tumors from normally fed controls.

The rate of tumor growth as measured by external calipering was the same in the scorbutic and control groups for a fortnight, after which the tumors in scorbutic guinea pigs grew much more slowly. The average weight of tumors removed from 14 scorbutic and moribund animals was 7.6 gm., whereas tumors taken concurrently from 7 normal animals had an average weight of 29.6 gm. This difference was found to be statistically significant ($p < 0.01$).

DESAMIDATION OF GLUTAMINE AND ASPARA-GINE IN NORMAL AND NEOPLASTIC HE-PATIC TISSUES. MAURICE ERRERA (by invitation) and JESSE P. GREENSTEIN. (National Cancer Institute, Bethesda, Md.)

Fetal rat liver possesses little or no asparaginase activity but does possess a high glutaminase activity. In adult rat liver, the relative activity of these enzymes is reversed, the glutaminase activity being extremely weak and the asparaginase activity very high. When the adult liver becomes neoplastic, the fetal pattern is noted in the hepatoma, *i.e.*, a near-disappearance of asparaginase activity and concomitant rise in glutaminase activity. That a tumor may possess the metabolic characteristics

of the corresponding embryonic form is not surprising by now.

The rate of desamidation of glutamine and asparagine in homogenates of all three kinds of hepatic tissues is greatly increased by added pyruvate. The pyruvate is not consumed in the reaction, but plays the role of a cosubstrate. This effect of pyruvate is almost exclusively a property of the liver, and is not observed to any great extent in other normal tissues. The fact that it is noted in hepatomas but not in any other tumors of different histogenesis shows that in this respect the hepatoma bears the imprint of its tissue of origin, and suggests a chemical method of distinguishing hepatomas from other kinds of tumors.

COBALT INHIBITION OF TUMOR RESPIRATION AND PROTECTION BY HISTIDINE. JOHN HEARON, ARTHUR L. SCHADE, HILTON LEVY, and DEAN BURK. (National Cancer Institute, Bethesda, Md., and Overly Biochemical Research Foundation, New York, N. Y.)

Cobalt has been shown previously to inhibit tissue respiration at concentrations of approximately 5 to 50 p.p.m. The inhibition is progressive with time, and tumor tissue is particularly sensitive. It has been found that the inhibition of tumor respiration may be prevented by additions of histidine at a molar ratio of histidine to cobaltous ion of 2 to 1 or greater. This protection is explicable on the basis of the reversible formation of a 2:1 histidine-cobalt complex, cobaltodihistidine, whereby the equilibrium constant, $K = \text{(cobaltodihistidine)} (H+)^2/\text{(histidine)}^2 (Co++) = 7.5 \times 10^{-7}$ at 38° C. The degree of protection afforded by a given concentration of histidine at any level of cobalt concentration may be correlated with the degree of completion of the reaction, and the observed inhibition in the presence of histidine is in accord with the concentration of free cobaltous ion calculated from the equilibrium expression. It may be concluded that the cobaltodihistidine is nontoxic. The rather slight protection given by other alpha amino acids and histamine parallels the observed lower coordination affinities of the compounds for cobaltous ion, except in the case of cysteine which forms a 3 cysteine: 1 cobaltous complex of high affinity that is rapidly oxidized to the cobaltic state and affords considerable protection. The progressive inhibition of tumor respiration by cobalt can only be halted but not reversed by additions of histidine. Thus, it appears that the combination of cobalt with the tissue component concerned is essentially irreversible, even in the presence of histidine. Further studies on the mechanism are in progress.

PURIFICATION AND PROPERTIES OF DEHYDROPEPTIDASES FROM NEOPLASTIC AND NORMAL TISSUES. JOSEPH SHACK. (National Cancer Institute, Bethesda, Md.)

Previous investigators have postulated from tissue distribution studies the existence of a dehydropeptidase I splitting glycyldehydroalanine with a uniformly high activity in tumors and of a dehydropeptidase II splitting chloracetyldehydroalanine and absent in tumors.

A purification and study of these enzymes from rat tissues has been carried out. By differential centrifugation at 3,000 and 18,000 R.P.M., it was found that the bulk of dehydropeptidase I of kidney is firmly bound to particulates sedimentable only at high speeds. In contrast the dehydropeptidase I of liver and tumor and the dehydropeptidase II of liver and kidney remain in the supernant. A hundred-fold concentration of dehydropeptidase I free of dehydropeptidase II has been achieved by differential centrifugation, enzymatic digestion and salt fractionation of kidney extract. The soluble enzymes have been purified by low temperature alcohol fractionation. The separation of activities made in the fractionation procedures confirm the existence of 2 distinct enzymes. They have been compared with respect to pH dependence, kinetics and specific inhibition. Neither is inhibited by azide, iodide or fluoride. Both are inhibited by cyanide and thioglycolate, inhibitions reversible on dialysis. Iodoacetate inhibits dehydropeptidase II (also reversed on dialysis) but has no effect on dehydropeptidase I.

These results indicate that the absence of dehydropeptidase II activity in hepatoma is due not to a change in specificity but to the disappearance of the enzyme as a result of malignancy. Comparative studies of purified dehydropeptidase I from liver and hepatoma have shown no differences in catalytic properties.

PHOSPHORYLATED INTERMEDIATES IN TUMOR GLYCOLYSIS. G. A. LePAGE. (McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.)

The rate of glycolysis has been reported to be high in tumors. There has been considerable controversy as to whether this was a phosphorylative or non-phosphorylative glycolysis. While data available can all be reasonably explained on the basis of a phosphorylative glycolysis and the enzymes necessary all appear to be present in tumors, the question of what type of glycolysis is operative had not been conclusively settled.

In this investigation analyses were made, using methods established as adequate for other tissues, for the intermediates of the Meyerhof phosphorylative glycolysis system. Tissues were fixed in liquid air. Components analyzed for included inorganic phosphorus, the adenine nucleotides, phosphocreatine, glycogen, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, hexosediphosphate, phosphoglyceric acid, phosphopyruvic acid and lactic acid. Analyses were confirmed in certain cases by isolation in high yield. Tumors so studied included several transplantable and certain primary rat and mouse tumors, and human carcinoma.

Glycogen was found to be low except in the human tumor samples. Lactic acid was elevated several fold above that of differentiated tissues in all cases. The levels of other intermediates conformed with those found in differentiated tissues. Modification of the physiological state of certain tumors by production of anoxia or hyperglycemia gave changes which were interpretable on the basis of a phosphorylative system.

THE DPN-CYTOCHROME REDUCTASE CONTENT OF CANCER TISSUE. M. RHIAN and VAN R. POTTER. (McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.)

The enzyme that has been referred to as DPN-cytochrome reductase catalyzes the reaction between diphosphopyridine nucleotide (DPN, coenzyme I, cozymase) and cytochrome *c*. When this enzyme is functioning, the substrate-hydrogen from the DPN-linked dehydrogenases is transported to oxygen via the cytochrome system.

Using the malic dehydrogenase system as a source of reduced DPN it has been possible to devise an assay system for the determination of DPN-cytochrome reductase. Assays have been carried out on normal rat liver, heart, kidney and brain tissue; and Walker carcinoma 256, Jensen sarcoma, Flexner-Jobling carcinoma and primary hepatomas, all from rats. It has been shown that the cancer tissues are extremely low in this enzyme as compared with the normal tissues studied thus far.

The results can be interpreted in terms of the balance between the glycolytic and the oxidative enzymes, since a relative deficiency of this enzyme would diminish the rate of oxidation of reduced DPN by oxygen, leaving it to be oxidized by pyruvate, which in turn would be converted to lactate. Thus a deficiency in cytochrome reductase could result in the glycolytic type of metabolism that is found in tumors.

SUCCINOXIDASE STUDIES OF THE LIVER CELLS OF MICE FED CARBON TETRACHLORIDE. KRETCHMER, N. (by invitation), TSUBOI, K. K. (by invitation), and BARNUM, C. P. (Department of Physiological Chemistry, University of Minnesota, Minneapolis, Minn.)

Alterations in the succinoxidase system that took place during the period of carbon tetrachloride feeding were presented from studies on cytoplasmic fractions of the mouse liver cell.

Liver tumors were induced in C3H mice over a period of 200 days by feeding 0.1 cc. of 40 per cent carbon tetrachloride in olive oil every 4 days. Animals were sacrificed at intervals for the succinic oxidase assay. The assays were conducted on the cytoplasmic particulates obtained by methods of differential centrifugation.

During carbon tetrachloride poisoning of the mouse liver, there is initially a decrease in the cytoplasmic succinoxidase activity of the liver cell; as the induction proceeds, the enzyme activity reaches normal values and exceeds the normal in the 60, 75, and 90 day periods. After 90 days the enzyme activity follows a downward trend such that at 200 days and at tumor, the enzyme activity is somewhat below the normal.

THE INTERFACIAL DENATURATION OF PROTEINS IN THE PRESENCE OF AROMATIC DIAMIDINES AND NUCLEIC ACIDS. M. J. KOPAC. (Department of Biology, Washington Square College of Arts and Sciences, New York University, New York, N. Y.)

These experiments augment the work reported at the A. A. A. S.-Gibson Island Conference of 1946 (*Cancer Research* 7:44-46. 1947). The effects of stilbamidine, propamidine, and *bis*-amidinomethyldibenzyl on the denaturation of bovine plasma albumin and of crystalline ribonuclease (Kunitz) at oil-water interfaces were measured.

The interfacial denaturation of albumin (2 mgm./ml.) was enhanced by stilbamidine (0.001M), less so by propamidine (0.001M), and completely inhibited by *bis*-amidinomethyldibenzyl (0.001M). With an albumin concentration of 5 mgm./ml., or higher, only stilbamidine enhanced surface denaturation, whereas others depressed it.

On adding Na zymonucleate (1 mgm./ml.) to the albumin-diamidine preparations, the diamidines were nearly completely antagonized. The combination of stilbamidine + propamidine, each at 0.001M, produced a typical stilbamidine effect. On adding Na zymonucleate (1 mgm./ml.) to this preparation, the action of stilbamidine was abolished and a typical propamidine effect was elicited, indicating that stilbamidine was preferentially bound by the nucleic acid. Stilbamidine was partly neutralized by yeast adenylic acid (1 mgm./ml.).

The interfacial denaturation of crystalline ribonuclease (1 mgm./ml.) was strikingly enhanced by stilbamidine (0.001M) and by propamidine (0.001M) and completely prevented by *bis*-amidinomethyldibenzyl (0.001M). All diamidine effects were abolished on addition of Na zymonucleate (1 mgm./ml.). Sodium thymonucleate (1 mgm./ml.) was less active than Na zymonucleate in abolishing the stilbamidine effect. Stilbamidine was not neutralized, however, if the Na zymonucleate was previously incubated with ribonuclease for 2 to 4 hours. Following incubation of Na thymonucleate with ribonuclease, stilbamidine was considerably neutralized.

These data indicate that certain diamidines enhance interfacial denaturation because they weaken side-chain linkages in protein molecules. No appreciable increase in interfacial denaturation was observed if these diamidines were removed before exposing the proteins to interfacial forces. The increased denaturation, therefore, results from the simultaneous action of surface forces with the diamidines.

Stilbamidine in the presence of other diamidines was preferentially bound by nucleic acids. These data may explain why stilbamidine produced the drastic action on the nucleoproteins tested to date. This compound, a denaturating adjuvent, is readily bound by nucleic acids.

LARGE SCALE PREPARATION OF THE TUMOR-NECROTIZING POLYSACCHARIDE FROM *S. MARCESCENS*. ADRIAN PERRAULT and M. J. SHEAR. (National Cancer Institute, Bethesda, Md.)

The cultivation of strain 724 of *S. marcescens* in a synthetic medium is being carried out in lots of up to 350 l. each. The organisms are separated, and the active agent in the filtrates concentrated to 1/200 of the original volume.

After further concentration and purification, bioassays for potency are carried out in mice bearing sarcoma 37. The potency of the final product is similar to that obtained in the small-scale preparations of the active polysaccharide previously obtained with culture filtrates from the "G. W." strain.

Active fractions have been obtained from the organisms themselves, and these fractions are being subjected to further purification. Yields are now measured in grams.

TUMOR NECROTIZING BACTERIAL POLYSACCHARIDE TAGGED WITH RADIOACTIVE IODINE. ARNOLD M. SELIGMAN, JOSEPH LEITER, BENJAMIN SWEET, and M. J. SHEAR.
(Surgical Research Department, Beth Israel Hospital, and Department of Surgery, Harvard Medical School, Boston, Mass., and the National Cancer Institute, Bethesda, Md.)

The polysaccharide from *Serratia marcescens*, which produces necrosis in tumors, was tagged with radioactive iodine (I^{131}). Unattached iodine was removed by dialysis.

Free iodine.—Iodination of 2.5 mgm. of polysaccharide with 0.25 mgm. of iodine resulted in the incorporation of 3 per cent of the iodine or 180 atoms of iodine per molecule of polysaccharide (approximate molecular weight 8,000,000). Ethylene linkages presumably were involved in the reaction. Some loss in tumor necrotizing potency was observed in mice bearing sarcoma 37.

Sodium hypoiodite.—Iodination of 2.5 mgm. of polysaccharide (P_3R) with iodine in the presence of sodium carbonate (10 mgm.) resulted in incorporation of the iodine. When 1.25 mgm., 0.25 mgm., and 0.05 mgm. of iodine were used, 0.7 per cent, 3.5 per cent, and 14.4 per cent of the iodine respectively were attached; the number of atoms of iodine per molecule of polysaccharide attached was 226, 223, and 183 respectively. The polysaccharide molecule, therefore, was readily saturated within a wide range of iodine concentration. Hydrogen, alpha to a carbonyl group, presumably was replaced in this reaction. No loss in tumor-necrotizing properties was observed in mice with sarcoma 37.

Mandler candle (1 in. long) filtration of a solution of tagged polysaccharide (25 μgm. per ml.) resulted in a 20 per cent loss of radioactivity.

Blood disappearance curves in mice, rabbits and man showed 40 per cent loss of radioactivity in 10 minutes and 75 per cent loss in 30 to 60 minutes.

The ratios of the radioactivity of tissues to that of circulating blood, when normal mice were sacrificed 1 hour after the injection of 25 μgm. of iodo-polysaccharide were, liver 1.3; lung 0.44; kidney 0.30; and thyroid 0.30. The ratios of the radioactivity of liver and tumor to that of circulating blood, when mice bearing sarcoma 37 were sacrificed after injection of 12.5 μgm. of iodo-polysaccharide, were as follows:

	Liver	Tumor
At 1 hour	2.8	0.23
At 14 hours	1.4	0.22
At 24 hours	4.9	0.43

SOME EFFECTS OF IODINATED BACTERIAL POLYSACCHARIDE ON PATIENTS WITH MALIGNANT TUMORS. THEODORE SACK, and ARNOLD M. SELIGMAN.
(Surgical Research Department, Beth Israel Hospital, and Department of Surgery, Harvard Medical School, Boston, Mass.)

Iodinated tumor-necrotizing polysaccharide from *S. marcescens* was administered intravenously to 7 patients with metastatic carcinoma. Individual doses varied between 2 and 3,000 μgm., repeated doses were given at no less than 24 hour intervals, and the maximum total dose to a single patient was 3,405 μgm. With some individual variations the post-injection reactions followed a common pattern, namely, a chill, with a subsequent temperature rise sustained for 1 or more hours, then a gradual fall to normal 12 to 30 hours after injection.

The blood pressure uniformly rose during the chill phase, began to fall when the temperature was maximum, and reached minimum levels between 8 and 15 hours after injection. Vigorous anti-shock therapy was necessary in 4 instances. Pulse and respiratory rates roughly followed the temperature curve.

The symptoms were those of a severe pyrogenic reaction with some gastrointestinal hyperactivity. Two patients suffered asthmatic dyspnoea; another developed mild congestive heart failure after the fourth injection. Albuminuria and leukocytosis were noted in all instances. Oliguria occurred only during periods of hypotension; persistent renal shutdown did not occur. Considerable tolerance to the toxic action of the material was developed in all patients.

Reactions following injection of iodinated polysaccharide did not differ appreciably from those following administration of the untreated material. The persistent post-injection exhaustion noted in many patients was greatly reduced in 2 patients by the administration of adrenal cortical extract both before and during the injection of iodinated polysaccharide.

THE EFFECT OF SIMULTANEOUS ADMINISTRATION OF BACTERIAL POLYSACCHARIDE AND ADRENAL CORTEX EXTRACT ON CELLS OF MOUSE TUMORS AND ON THE ADRENAL GLANDS OF THE HOST. IRENE COREY DILLER.
(Institute for Cancer Research, Lankenau Hospital, Philadelphia, Pa.)

In an attempt to alleviate some of the toxic effects of the tumor-necrotizing polysaccharide of Shear, Upjohn adrenal cortical extract and polysaccharide were administered simultaneously to mice (Beck and Diller, unpublished data). This did not inhibit the action of the polysaccharide on tumor tissue (sarcoma 37). The process of tumor cell degeneration, however, differed from that obtained following polysaccharide alone, and the responses occurred more slowly, reaching a maximum at about 24 hours instead of at 6. Marked fragmentation of tumor cells was apparent and unfragmented nuclei were condensed and crenulated. Acetic orcein stains these tumors very hazily and the cytoplasm is grayish and opaque, which suggests some chemical as well as structural

change. Cellular changes occur also in adrenal glands of the host, particularly in the medulla, when polysaccharide only (0.01 mgm.) or Upjohn extract only (0.5 cc. in 5 single doses) are injected intraperitoneally. Damage to the adrenal cell appears to be largely overcome when the 2 are given simultaneously.

COMPARATIVE STUDIES OF THE IMMUNOLOGICAL, TOXIC, AND TUMOR-NECROTIZING PROPERTIES OF *S. MARCESCENS* POLYSACCHARIDES. HUGH J. CREECH, MARY ALICE HAMILTON, IRENE COREY DILLER, EDWIN T. NISHIMURA, and M. J. SHEAR. (Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pa., and the National Cancer Institute, Bethesda, Md.)

Effective clinical utilization of the tumor-necrotizing property of *S. marcescens* polysaccharide has been hindered by the concomitant toxic and immunological properties. Although these 3 properties appeared to run parallel in the earlier preparations of this polysaccharide, fractionation of one preparation yielded products in which the toxic and antigenic properties were decreased considerably, whereas the tumor-necrotizing property was not altered significantly.

Studies have been made of the effects of passive immunization of mice against the toxic action of the polysaccharide from the "G.W." strain using the γ-globulin fraction of rabbit antisera. These antibody-containing fractions upon injection into normal mice a few hours before the administration of a lethal dose of polysaccharide protected a high percentage of the animals. Injection of the γ-globulin fractions into mice bearing sarcoma 37 prior to the injection of relatively large tumor-necrotizing doses of polysaccharide afforded definite protection against the lethal action but did not seem to interfere significantly with the tumor-necrotizing action of the polysaccharide.

Two recent preparations of polysaccharide from a different strain (#724) of *S. marcescens* have been found to be less antigenic and less toxic that the preparations from the "G.W." strain; in addition, they are not related antigenically to the latter. The influence of the γ-globulin fraction of antisera toward these preparations on the toxic and tumor-necrotizing actions of the polysaccharide is being investigated.

EFFECT ON SARCOMA 37 IN TISSUE CULTURE OF TWO TUMOR-NECROTIZING AGENTS. JANE R. McCONNELL, SUZANNE F. HALLETT and M.J. SHEAR. (Institute for Cancer Research, Philadelphia, Pa., and the National Cancer Institute, Bethesda, Md.)

The action in tissue culture of a preparation of the polysaccharide from *S. marcescens* and of emetine hydrochloride was studied. Each of these agents, when injected into mice bearing sarcoma 37, produces necrosis in the tumor.

Hanging drop cultures of sarcoma 37, grown for 18 hours, were treated directly with emetine hydrochloride

(concentrations of 10 mgm./cc. to 0.00001 mgm./cc). T cells became rounded; blebs formed and were pinched o nuclei shriveled and became pycnotic. The speed action and the degree of destruction of the cells in cultu were proportional to the concentration of the emeti hydrochloride. Control experiments showed that t damage was not attributable to the hydrochloric aci The damage seemed to be correlated with the appare surface reducing effect of emetine on the nucleus a cytoplasm.

When hanging drop cultures of sarcoma 37 we similarly treated with the polysaccharide (concentratio of 10 mgm./cc. to 0.001 mgm./cc.), however, no necrot ing effects were observed, even though these concentr tions regularly produced hemorrhage and necrosis *in vi*

The tissue culture method is of value in studies on t mechanism of action of such chemical agents. Howeve it is clear that, while it may give evidence of a dire effect of some substances on normal and malignant ce in cultures, it may not be entirely dependable if employ as a screening procedure in a chemotherapy program lieu of *in vivo* screening.

CHEMOTHERAPY OF CANCER. CLASSES C COMPOUNDS UNDER INVESTIGATION AN ACTIVE COMPONENTS OF PODOPHYLLI JONATHAN L. HARTWELL (by invitation), a M. J. SHEAR. (National Cancer Institute, B thesda, Md.)

More than 1,200 organic compounds have been : sembled for screening in tumor-bearing animals. Amo the classes of compounds that have been obtained a alkaloids; isoquinolines; derivatives of phenylethylamir phenylpropylamine and phenylisopropylamine; car amates; azo compounds; derivatives of phenanthrer acenaphthene, and fluorene; diphenylethanes; stilben a, β-unsaturated ketones and quinones; acridin quaternary ammonium salts; sulfonamides; mercuria arsenicals; and substances known to affect intermedia metabolic processes.

About 500 of these compounds have been put throu a preliminary first screening in mice bearing sarcoma : With a few compounds, more extensive biological wo has been done. The exploratory screening indicated th some classes of compounds contain a higher percenta than others of members capable of inducing damage tumor tissue under the conditions of these experimen For example, of 20 isoquinolines screened, only 1 ga microscopic evidence of obvious damage as compar with: 10 of 38 acridines; 23 of 211 quaternary ammoniu salts; 6 of 34 arsenicals; 8 of 53 alkaloids; 4 of 53 β-diphenylethylamines; 1 of 11 sulfonamides; 2 of stilbenes; and 1 of 10 phenanthrene derivatives.

Podophyllin produced severe gross damage in the t mors. Fractionation yielded 2 white crystalline cor pounds, podophyllotoxin and a new substance designat provisionally as NCI-1074. Each of these 2 compoun possessed tumor-damaging properties in a single do down to 3 μgm. per gm. body weight. Quercetin, anoth crystalline podophyllin component, yielded negative r sults at ten times this dose. The possible presence

other active constituents is under investigation. Picro-podophyllin, prepared from podophyllotoxin, gave negative results in doses up to 12 μgm. per gm. body weight.

NCI-1074 is isomeric with podophyllotoxin and picro-podophyllin, has a melting point close to that of picro-podophyllin, but is identical with neither.

HISTOLOGIC CRITERIA FOR EVALUATING THE CAPACITY OF CHEMICAL AGENTS TO PRODUCE DAMAGE RAPIDLY IN SARCOMA 37. ROSS C. MacCARDLE and VIRGINIA DOWNING. (National Cancer Institute, Bethesda, Md.)

The necrosis-producing capacity of chemical agents injected in single doses subcutaneously into mice bearing intramuscularly implanted sarcoma 37 was ascertained histologically by observing the extent and speed of changes in cells of tumor and intestinal epithelium fixed in Zenker's formol-bichromate fluid at 8, 20 and 48 hours after administration. No regression experiments were attempted in this preliminary screening of many compounds. Control tumors showed resting and dividing cells with varying amounts of spontaneous degeneration. One feature of old necrosis is the presence of extracellular bluish debris.

Tumors treated with some compounds showed extensive degeneration and necrosis in which moribund processes seemed to be in approximately the same stage, suggesting simultaneous induced injury. *Compound 368,* N-acetyliodocolchinol methyl ether, attacked tumors apparently directly, arresting mitoses in metaphase followed by necrosis. *Compound 707,* a quaternary ammonium salt, attacked tumor cells evidently directly and indirectly after vascular damage. *Compound 497,* α-phenyl-β-(3,5-diiodo-4-hydroxyphenyl)-proprionic acid, induced necrosis in some tumors. Tumors, in treated mice, showing necrosis sharply demarcated from healthy tumor tissue were considered unaffected, since control tumors occasionally presented this appearance tentatively attributed to localized spontaneous vascular blockage. *Agent 85V,* podophyllin, induced cell damage throughout the tumor apparently directly and indirectly with marked stasis and blood vessel damage. Intestinal and some tumor cells were arrested in various stages of mitosis. Mouse epidermis painted with podophyllin showed many large clear cells and polymorphic nuclei in atypical mitosis; while the intestine of the same animal showed arrested mitoses. Rous sarcoma in chickens treated with podophyllin showed induced necrosis; and cerebellar Purkinje cells were also damaged. Cell death is being studied by silver, orcein, Masson, microincineration and phase-contrast methods in these and other tumors.

THE EFFECT OF PODOPHYLLIN ON TUMOR CELLS *IN VITRO.* RICHARD A. ORMSBEE and IVOR CORNMAN. (Sloan-Kettering Institute for Cancer Research, New York, N. Y.)

A sterile suspension of crude podophyllin, when incorporated into the nutrient medium in roller tube tissue culture preparations, exerts a toxic and repressive effect against tumor cells from the in-strain transplantable mouse tumors, sarcoma L946 A II and lung tumor MA 387. The effect on normal mouse embryonic skin growing in the same tube is negligible at concentrations which cause extensive tumor cell damage. This differential toxic effect is more marked than that obtained with any of the other known mitotic poisons which have been tested so far. This material is now being tested *in vivo* for repressive effect against a variety of tumors.

TRYPANOSOMA CRUZI IN THE TREATMENT OF MOUSE TUMORS. THEODORE S. HAUSCHKA. (Institute for Cancer Research, Lankenau Hospital, Philadelphia, Pa.)

The work of Roskin and his collaborators on destruction of tumors in animals by *Trypanosoma cruzi* "endotoxins" has recently been extended to the clinical application of Klyueva's "cancerolytic" *T. cruzi* lysates and has given impetus to related studies. Our experiments were started in March 1945 under a joint institutional program participated in by the Chemotherapy Section of the National Cancer Institute and the Laboratory of Zoology (National Institute of Health) and the Lankenau Hospital Research Institute.

Infections of *Trypanosoma cruzi* ("B"-strain) significantly retarded the growth of three transplantable tumors: squamous epithelial carcinoma 119, mammary adenocarcinoma, and sarcoma 37. Spontaneous breast adenocarcinoma in C3H mice was slightly retarded in growth.

The inhibitory effect was often accompanied by loss in body weight and parasitemia of vital organs. Of the 4 tumor varieties studied, only carcinoma 119 was found to be parasitized. Cancer cells proper were rarely invaded by *T. cruzi,* but parasites were relatively abundant in the stroma and in the encapsulating connective tissue. Retardation of tumor development did not result in longer survival.

Growth of carcinoma 119 was retarded or completely inhibited by infection with (1) the lethal "R-strain of *T. cruzi* (obtained from the same source as Roskin's strain); (2) a mixture of 5 avirulent strains ("A"-, "M"-, "P"-, "T"- and "C"-strain); (3) the entirely avirulent "C"-strain. Tumor-inhibition by "C"-strain was *not* accompanied by loss in body weight or other symptoms of Chagas' disease, and infected tumor-bearing mice lived longer than tumor controls.

Growth of spontaneous mammary adenocarcinoma (C3H mice) was inhibited by infection with "R"-strain *T. cruzi.* This otherwise lethal infection can be cured by treatment with the quinoline derivative, Bayer 7602.

Heat-killed cultures (50° C.) and lysates of *T. cruzi* ("B"-strain) were without effect against carcinoma 119 or mammary tumors. A lysate prepared from "R"-strain of *T. cruzi* in the plasma of infected mice contained a tumor-necrotizing "endotoxin" but also produced degenerative symptoms in liver, spleen and kidney. Test mice treated with this lysate died earlier than the controls.

THE EFFECT OF INHIBITORS OF INTERMEDIARY METABOLISM ON ADVANCED HUMAN NEOPLASIA. MAURICE M. BLACK

and ISRAEL S. KLEINER. (New York Medical College, New York, N. Y., and Brooklyn Cancer Institute, Brooklyn, N. Y.)

Malignant tissues have long been known to exhibit greater aerobic and anaerobic glycolytic activity than homologous normal tissues. Attempts to inhibit the growth of such tissues by the use of inhibitors of glycolysis by other investigators have yielded indecisive results. In view of the importance of the active phosphate bonds in energy-yielding reactions, we have attempted selective inhibition of such reactions in relation to these bonds.

The inhibitors used were sodium fluoride, iodoacetic acid, malonic acid and sodium azide. In the doses used, these substances, both singly and in combination, resulted in encouraging therapeutic effects without evidence of appreciable toxicity. The 31 cases studied, all far advanced, included acute leukemia and a diversified group of malignant tumors. Hematological and clinical remissions, for a period of 3 months, were observed in a significant number of leukemias studied. The beneficial results in patients with various types of malignant tumors included shrinkage of tumor mass, relief of pain, increase in weight and well-being, and degenerative changes in material obtained in repeated biopsies in 1 case of lymphosarcoma.

In this work, adaptation to such agents seems to be the limiting factor in continued therapeutic effect. Thus, after refractoriness to sodium fluoride and iodoacetic acid had developed, a therapeutic effect was obtained by the addition of malonic acid. Reversal of the refractory state with renewed sensitivity to the glycolytic inhibitors was also accomplished by the use of sodium azide.

The findings reported would appear to be consistent with the hypothesis of the importance of the active phosphate bond and of the possible role of accessory pathways in this phenomenon. This and other hypotheses are under investigation.

CHANGES IN THE REDUCING POWER OF PLASMA IN PATIENTS WITH MALIGNANT NEOPLASIA AND THERAPEUTIC IMPLICATIONS. MAURICE M. BLACK. (New York Medical College, New York, N. Y., and Brooklyn Cancer Institute, Brooklyn, N. Y.)

Determination of the reducing power of plasma (or serum) was made by the use of the redox dyes, brilliant cresyl blue and methylene blue. It was found that plasma of patients with malignant diseases tended to have a lowered reducing power and could usually be distinguished from normal plasma and from plasma of patients suffering from conditions other than malignancy. The decreased reducing power obtained with plasma from cancer patients tended to be grouped at different levels with individual sites of tumor origin. So far, in advanced pregnancy and in advanced hepatic cirrhosis, similar decreased reducing power has been observed. Adequate therapy (x-ray or surgery) increased the reducing power. Thus, this effect of therapy could be followed objectively.

The correlations between the results of this procedure and the diagnoses are illustrated by the following ratios.

The numerator represents the number of cases showing good correlation between the clinical pathological diagnoses and the alteration in reducing power; the denominator gives the total number of cases involved. Controls (normals) 50/50; nonmalignant diseases 111/120; active malignant disease 158/184.

These observations suggested the concept that some of the symptomatology associated with malignancy might be due to the altered enzyme activity as a result of diminished -SH potential. Accordingly, glutathione or cysteine was administered intravenously. This was followed rapidly by relief of pain and general symptomatic improvement. No effect was noted, however, on the growth rate of the tumor itself.

IN VIVO STAINING OF MALIGNANT TISSUE IN MICE. MARGARET REED LEWIS and PHILIP P. GOLAND. (The Wistar Institute of Anatomy and Biology; Department of Neurosurgery, Hospital of the University of Pennsylvania; and the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pa.)

Certain dyestuffs, namely, 3 oxazine, one thiazine, 4 xanthene, one acridine and 1 anthraquinone dye added to the diet of tumor-bearing mice stained the tumors selectively. The *in vivo* staining was accompanied by retardation of their growth.

Twenty-five dyestuffs were pulverized with fox chow in amounts equivalent to 0.4 per cent of commercial and 0.15 per cent of other compounds. Thirty mice of an inbred strain were then implanted subcutaneously with a small graft of a sarcoma native in the strain used, placed in individual containers and given 10 grams of food each day; 25 of them receiving treated and 5 untreated fox chow. Samples of urine were examined, and the mice were sacrificed about 14 days later, by which time those receiving untreated food had large tumors. As each mouse was sacrificed records were made of its condition and of the size and color of its organs and tumor. If the mouse was normal and its tumor large and uncolored the test was not repeated. If, however, its tumor was small and stained, the test was repeated. Tests on mice that became ill were repeated using less dyestuff. Compounds that stained and retarded sarcomas were tested also on mice bearing spontaneous adenocarcinomas.

Our results show that compounds that stain and retard tumors have certain structural similarities. Further investigation should disclose the structural nature of compounds less toxic and more selective in their inhibiting action on malignant tissue so that they can be synthesized for use.

STUDIES ON PURIFICATION OF THE AGENT OF CHICKEN TUMOR I. W. RAY BRYAN and VERNON T. RILEY. (National Cancer Institute, Bethesda, Md.)

Progress has been made toward purification of the agent of chicken tumor I in experiments carried out with quantitative biological assays, quantitative nitrogen determinations, and electron microscopic observations. The results of investigations involving the principles of

ultracentrifugal fractionation and chromatography were described.

RELATIONSHIP BETWEEN THE LETHAL YELLOW (A^y) GENE OF THE MOUSE AND SUSCEPTIBILITY TO SPONTANEOUS PULMONARY TUMORS. MARGARET K. DERINGER and WALTER E. HESTON. (National Cancer Institute, Bethesda, Md.)

Susceptibility to induced pulmonary tumors has been shown to be associated with the lethal yellow gene (A^y) of the mouse. Yellow F_1 hybrid mice from a cross between strains A and Y were more susceptible to pulmonary tumors induced by 20-methylcholanthrene than were their brown litter mates. In the present experiment the data indicated a relationship between the lethal yellow gene and spontaneous pulmonary tumors.

Eighty-two AYF_1 hybrids were produced and were autopsied at 15 months of age. Sixteen of the 38 yellow mice and 9 of the 44 brown mice had pulmonary tumors. The results suggested a higher degree of susceptibility in the yellow mice but were not significant. A second group of 83 mice was therefore produced and was autopsied at 15 months of age. Fifteen of the 38 yellow mice and 10 of the 45 brown mice had pulmonary tumors. These results were not significant but the combined results for the 2 groups were highly significant, $X2 = 7.425$; $P <0.01$.

The previously demonstrated effect of the A^y gene on body size was shown by the weights of the second group at 6, 12, and 15 months of age. At 6 months the average weight for the yellow males was 14 gm. higher than that for the brown males; and that for the yellow females was 19.6 gm. higher than that for the brown females. This difference was approximately halved at 12 months and at 15 months the yellow and brown mice were of approximately equal weight.

MORPHOGENESIS AND EVOLUTION IN MALIGNANT TUMORS. SPONTANEOUS MATURATION AND REGRESSION OF TESTICULAR NEOPLASMS. NATHAN B. FRIEDMAN. (Army Institute of Pathology, Washington, D. C.)

Study of 1,000 tumors of the testis (*Mil. Surgeon*, 99: 573-593. 1946) has revealed that teratoid growths result from the maturation of originally undifferentiated neoplasms. It is suggested that primitive cells from such tumors may metastasize before differentiation takes place, a hypothesis which would explain why teratomas lacking histologically malignant components are sometimes associated with metastases.

Some trophoblastic tumors of the testis disappear completely despite progression of their metastases. The tendency toward vascular invasion, hemorrhage and necrosis and possibly the normally brief life span of trophoblastic tissue may account for such regression. The primary site of the neoplasm remains marked by a peculiar cicatrix, which, when overlooked, leads to the erroneous diagnosis of extragenital chorioepithelioma.

Regression is not restricted to trophoblastic tumors. The tuberculoid granulomas which are common secondary stromal components of germinomas (seminomas) sometimes become more prominent than the seminomatous tissue. It is difficult or even impossible at times to identify residual neoplastic elements when the bulk of the "tumor" is made up of lymphocytes, epithelioid cells, fibroblasts and giant cells.

Teratoid tumors may be governed by oncologic principles which do not apply to other types of neoplasms. However, it might be worth investigating the new growths of other organs for evolutionary and regressive tendencies comparable to those of testicular tumors. The factors controlling neoplastic maturation and regression and the possibility of influencing them should be explored.

GENETIC FACTORS AFFECTING SYNERGISM OF LEUKEMOGENIC AGENTS. HARRY W. MIXER and ARTHUR KIRSCHBAUM. (Departments of Radiology and Anatomy, University of Minnesota Medical School, Minneapolis, Minn.)

Mice of the dba strain (subline 212) are susceptible to the induction of leukemia by either x-rays or methylcholanthrene administered independently. Although strain CBA is very susceptible to the leukemogenic action of x-rays, this stock has proved to be absolutely refractory to the induction of leukemia by methylcholanthrene.

When treatment with x-rays (1,000 r in divided doses) was combined with 18 skin paintings of methylcholanthrene dissolved in benzene (0.25 per cent solution), the incidence of induced leukemia was increased (59 per cent with combined treatment, 34 per cent with x-rays only, 33 per cent with methylcholanthrene only). When subthreshold doses of the 2 agents were combined, absolute synergism was obtained (no induced leukemia with either 6 skin paintings of methylcholanthrene, or 200 r of x-rays used alone, but 30 per cent induced leukemia when the 2 agents were combined).

Although synergism could be demonstrated in dba mice where susceptibility to each leukemogenic agent was manifest, neither synergistic nor additive effects could be obtained by combined administration of these agents to CBA mice. The incidence of leukemia was the same if 500 r were given in divided doses either alone or in combination with 18 skin paintings of methylcholanthrene.

These results suggest that leukemogenic agents may act synergistically only if the test animals are susceptible to each of these agents independently.

CHEMICAL FACTORS CONCERNED IN THE MUTUAL ADHESIVENESS OF EPITHELIAL CELLS. IRVING ZEIDMAN. (Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.)

Experiments were designed to determine the chemical factors responsible for maintaining mutual adhesiveness of human squamous epithelial cells. The method used depended upon separation of pairs of cells by micromanipulation, the value of adhesiveness being determined by the bend produced in a calibrated microneedle when subjected to the strain of detaching the cells. Adhesiveness was decreased in the absence of calcium or magnesium, or both. Reduction in adhesiveness brought about in a calcium-free solution was not reversed by restoring

calcium to the medium. Excess of potassium in the solution did not alter adhesiveness. Decrease in adhesiveness was produced by methylcholanthrene, a substance reported to lower the calcium content of squamous epithelium. These results offer an explanation for changes in adhesiveness recently reported in cancer cells. In these malignant cells, adhesiveness was found decreased as compared with that of normal epithelium. Since the calcium content of cancer cells has been reported to be abnormally low, it is regarded as probable that lessened adhesiveness of cancer cells is explained by their deficiency in calcium.

CHANGES OF CARBOHYDRATE METABOLISM IN PATIENTS WITH GASTRIC CANCER AND IN MICE BEARING SARCOMA 180. J. C. ABELS, C. J. KENSLER, N. F. YOUNG, and F. HOMBURGER. (Sloan-Kettering Institute for Cancer Research, New York, N. Y.)

Studies of hepatic glycogen concentration in patients with gastric cancer and in controls undergoing laparotomies for benign abdominal disorders have shown that while the glycogen concentration is not different in the livers of patients with gastric cancer when the biopsy is taken after a 10 hour fast, it is considerably lower in the patients with cancer when both groups of patients receive glucose in the 10 hour period preceding the operation. This defect of glycogenesis can be corrected by the administration of adrenal cortical extract.

Studies on mice bearing transplanted sarcoma 180 revealed similar defects of hepatic glycogenesis. This metabolic defect is therefore independent of the type of tumor present and occurs, at least in the mouse, even when the tumor is located outside of the portal circulation.

RESPONSE OF MOUSE MYELOGENOUS LEUKEMIA TO URETHANE. ARTHUR KIRSCHBAUM and C. S. LU. (Department of Anatomy, University of Minnesota Medical School, Minneapolis, Minn.)

The administration of a single anesthetic dose of urethane resulted within 24 hours in a drop in the white blood cell count and the appearance of many mature cells in the bone marrow of mice with myeloid leukemia. The depression in white blood cell count continued until 72 hours after injection, following which the counts rose. However, they had not reached the initial level 6 days after the single injection in 8 of the 11 mice tested.

The ratio of segmented to mononuclear myeloid (blast) cells in the leukemic marrow ranged from 13:87 to 46:54, with an average of 26:74. This ratio was reversed within 24 hours following a single injection of urethane (1 mgm. per gm. of body weight in aqueous solution given IP).

The number of mitotic figures in the myeloid cells of leukemic marrow was decreased following the administration of urethane. Maturation may have been secondary to inhibition of mitosis in blast cells. However, in the treated mice there were fewer marrow cells capable of undergoing division, which may account for the reduced number of division figures.

It is suggested that the release of an increased percentage of mature cells into the circulating blood may be a factor in depression of white blood cell counts following the injection of urethane into mice with myeloid leukemia.

THE METABOLISM IN THE MOUSE OF 1, 2, 5,-6-DIBENZANTHRACENE LABELED IN THE 9-POSITION With C[14]. CHARLES HEIDELBERGER and HARDIN B. JONES. (Radiation Laboratory, University of California, Berkeley, Calif.)

Previous investigations of the metabolism of dibenzanthracene, using ultraviolet absorption spectroscopy as the analytical tool, has led to the isolation and characterization of 4′,8′-dihydroxydibenzanthracene and some preliminary information as to the distribution of this carcinogen in the animal body. This work was summarized by R. Norman Jones (*Cancer Research*, 2:237. 1942) who made a considerable contribution to this field, and who pointed out the difficulties inherent in this method of analysis.

Dibenzanthracene labeled in the 9 position with C[14] has been synthesized by Heidelberger, Brewer, and Dauben (*J. Am. Chem. Soc.* In press) and this material which has a specific activity 0.385 μc./mgm. is being used in an investigation of the metabolism and mechanism of carcinogenic action of this compound. Small doses of known radioactivity are administered in various ways to mice, which are kept in metabolism cages to recover the carbon dioxide of respiration as well as the urine and feces. The animals are dissected, the organs to be assayed are burned with oxygen in a combustion furnace, and the carbon dioxide is precipitated and counted in the form of barium carbonate with thin-window Geiger-Mueller counters. Dibenzanthracene has been administered to mice as a colloid in isotonic glucose, and in fat solutions.

The most striking fact observed in the metabolism of dibenzanthracene injected intravenously as an aqueous colloid, is the rapid elimination of large quantities in the feces.

A bile-fistula was performed on a mouse and after injection of the colloid, all detectable activity was present in the bile. Since there was no activity observed in the intestines or the intestinal contents, the excretion must be entirely through the bile. Chemical investigation of this bile reveals that the activity is due almost entirely to unaltered dibenzanthracene. In some cases there has been a small amount of radioactivity in the carbon dioxide of respiration, indicating the complete oxidation of at least one carbon in the molecule. This point is undergoing further investigation.

In general, it can be stated that the activity is not highly concentrated in any specific tissue of the body, but seems to be distributed fairly evenly throughout the internal organs. The mode of administration affects the amount absorbed in the body, exclusive of the gastrointestinal contents. At the end of 24 hours, 25 per cent of the activity was absorbed from intraperitoneal injection, whereas 5 per cent was absorbed from stomach tube and from intravenous injection. When the substance is given to animals bearing highly developed mammary

carcinoma, there is no appreciable concentration in the neoplasm. When the compound is administered intraperitoneally, either in oil or as an aqueous colloid, there is a higher concentration of activity in the intestines than in the intestinal contents. This is not observed with other methods of administration, and indicates the path of absorption to be across the exposed peritoneum of the gut. Subcutaneous administration in oil indicates that at the end of six weeks, 52 per cent of the dibenzanthracene is retained near the site of injection. However, a small tumor which appeared at the site in that time interval, showed that an appreciable part of the radioactive carbon in the tumor was no longer present in the form of dibenzanthracene, but 14 per cent has been converted into an acidic form, and 21 per cent to a phenolic form. Thus 65 per cent of the dibenzanthracene is unaltered.

Aliquots of an extract of this tumor were assayed by both possible methods. The amount of dibenzanthracene calculated from the spectrographic data was twice the quantity obtained by direct radioactive assay, and this justifies previous observations that spectrographically interfering substances other than the carcinogen tend to give unreliable results.

Work is now in progress in these laboratories on the mechanism of tumor production and regression with radioactive dibenzanthracene. Distribution studies over longer periods of time, using various modes of administration, are being continued with the ultimate aim of establishing, if possible, the site and mechanism of dibenzanthracene metabolism, degradation, and elimination in the mouse.

This paper is based on work performed under Contract = W-7405-Eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley, Calif.

CARCINOMA OF THE COLON IN RATS FOLLOWING THE FEEDING OF RADIOACTIVE YTTRIUM. HERMAN LISCO, AUSTIN M. BRUES, MIRIAM P. FINKEL, and WALTER GRUNDHAUSER. (Metallurgical Laboratory, University of Chicago, and Argonne National Laboratory, Chicago, Ill.)

Yttrium[91], one of the common radioactive fission products obtained in a chain-reacting pile, is a pure beta-emitter with an energy of 1.5 mev and a half-life of 57 days. It is essentially not absorbed and, since the material remains longer in the colon than in any other portion of the intestinal tract, most of the damage occurred in this region.

One group of rats received a single feeding by stomach tube of from 1.0 to 6.0 mc. of Y[91]. Of the 33 animals in this group, 4 died with adenocarcinoma of the colon. The earliest tumor was seen at 135 days and the latest at 506 days. Additional animals died with acute and chronic ulceration of the colon accompanied by benign and atypical hyperplasia of the mucosa.

A second group of rats was given 78 feedings of 0.46, 0.20, or 0.06 millicuries of Y[91] per feeding over a period of 3 months. The total accumulated doses were 31.20, 15.60, and 4.68 mc., respectively. Clinically all animals appeared well during the feeding period and growth was not impaired. Six of the 8 animals at the 2 higher levels died with carcinoma of the colon from 304 to 548 days after the first feeding. No malignancies were observed at the lowest level. However, many of these animals died with superficial ulcerative lesions of the colon.

THE INFLUENCE OF COSMIC RADIATION ON THE INDUCTION OF CANCER. FRANK H. J. FIGGE. (University of Maryland Medical School, Baltimore, Md.)

The hypothesis that the action of carcinogenic substances may be related to their efficient conversion of some form of penetrating radiation such as cosmic radiation to a form of energy capable of inducing intracellular malignant transformations was suggested by previous work. To test this hypothesis, 182 mice of inbred strains were injected with 0.25 mgm. of methylcholanthrene. They were divided into two groups and subjected to different intensities of cosmic radiation. One group consisting of 69 mice in 3 aluminum cages, used as controls, received normal, unmodified sea-level cosmic radiation. The remaining 113 mice, in 5 aluminum cages with lead plate covers, were subjected to the normal sea-level cosmic radiation plus the showers of radiation resulting from passing cosmic radiation through 1 or 2 lead plates 1 cm. thick. The average latent period for carcinogenesis in the controls was 80 days. The average latent period for induction of sarcomas in the 113 experimental mice receiving the intensified cosmic radiation was only 60 days, or three-fourths that of the controls. A repetition of this experiment gave the same results. While these results are significant, experiments to test this hypothesis in a more conclusive manner are desirable, and are contemplated.

TISSUE ELEMENTS IN THE ORIGIN OF NEOPLASMS. EVIDENCE THAT NEOPLASMS ORIGINATE AT VARIOUS LEVELS OF TISSUE ORGANIZATION. ANDERSON NETTLESHIP. (Alexander Blain Hospital, Detroit, Mich.)

A number of early human carcinomas in various tissues were studied in order to determine the kind of immediate environment out of which cancer arises. These observations pointed to morphologic evidence that certain neoplasms have a multicellular origin. It was concluded that the majority of neoplasms originate in tissues that are in an involutionary phase and that have widespread atrophy of the parenchyma. Furthermore, such tissues may, in some areas, show hyperplasia. The degree of atrophy was usually severe. Also, a number of cases were studied that illustrate grossly and microscopically the qualitatively different type of organization manifested by neoplasms. Cases are given of (1) osteogenic sarcoma, (2) adenocarcinoma of the colon, and (3) melanocarcinoma. It is suggested that the degree of organization is dependent upon the level at which the tissue control is destroyed at the time the neoplasm is established. Another aspect, that of morphologic evidence that certain neoplasms have multicellular origin, was studied in carcinoma *in situ* of the breast, carcinoma *in situ* of the stomach, epidermoid carcinoma

of the skin and cervix uteri. In all of these it was possible to show in a focal area that the cells were approximately of the same age of neoplastic development. Additional data was submitted from tissue culture studies and induced carcinomas in lower species. In summary, it may be stated that this evidence points to the origin of cancer from tissues that have widespread atrophy. The type of cancer that is established morphologically depends upon the level at which the tissue organization breaks down.

GENETIC AND ENDOCRINE FACTORS IN ADRENAL CORTICAL TUMOR FORMATION. GEORGE W. WOOLLEY and MARGARET M. DICKIE. (Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

There are important genetic factors behind the occurrence of adrenal cortical tumors. This is indicated by pronounced strain differences in response to gonadectomy. The adrenal cortex of strain JAX C57 brown, for example, undergoes only slight change in size or structure following gonadectomy. Strain JAX dba develops nodular hyperplasia of the adrenal cortex and strain JAX ce, adrenal cortical carcinoma. Hybridization experiments now in progress also point to the importance of the genetic factors.

Endocrine factors are also of great influence. In our series, adrenal cortical tumors of the types described in this report did not occur without gonadectomy. Following gonadectomy certain endocrine preparations will prevent their occurrence in a genetically susceptible strain. It is known that there is an intimate relationship between the adrenal cortex and the pituitary. In these first experiments the relationship is evidenced by occasional abnormalities of the anterior lobe of the pituitary in the experimental and not in the control series.

It seems evident that there is a relationship between the adrenal cortex and the gonad, and probably between gonadotropic and adrenocorticotropic factors. An hypothesis is that with the gonad absent there is increased need of activating materials which the adrenal cortical cells may be at least partially equipped to supply. The extent to which these are needed and/or the ability of the cells to fulfill the need is undoubtedly under genetic control.

THE EFFECT OF CALORIC RESTRICTION ON THE INCIDENCE OF MAMMARY TUMORS IN CASTRATE HORMONIZED C3H MICE. CARMEN B. CASAS, JOSEPH T. KING, and M. B. VISSCHER. (Department of Physiology, University of Minnesota Medical School, Minneapolis, Minn.)

Thirty C3H mice were ovariectomized at 21 to 23 days of age. They were divided into two groups of 15 each. One group was fed *ad libitum*; the other was restricted 33 per cent in caloric intake. Both groups were fed 0.5 gamma of diethylstilbestrol daily, and both received the same absolute amounts of protein, minerals and vitamins.

Vaginal smears made by the lavage method showed a constant dense, mixed-cell picture with predominance of cornified cells in both control and experimental groups, with no recognizable difference between the two.

At the time the first tumor appeared in the control group, accidents and sacrifice of animals for tissue study had reduced the control group to 13 animals and the restricted to 10.

In the controls 3 tumors appeared in the 24th week after ovariectomy; 2 in the 27th; 1 in the 28th; 2 in the 31st and 3 in the 33rd week. Only 2 controls are tumor-free at the end of the 39th week.

Only 1 tumor has been found in the restricted animals; it appeared in the 38th week. Evidently caloric restriction did not influence the vaginal response to estrogen in these animals but did significantly alter the tumor age.

THE MILK AGENT. SAMUEL GRAFF, DAN H. MOORE, WENDELL M. STANLEY, HENRY T. RANDALL, and CUSHMAN D. HAAGENSEN. (Columbia University, and Rockefeller Institute for Medical Research, New York, N. Y.)

This communication reports our progress toward isolation and characterization of the mouse mammary carcinoma agent transmitted by and present in the milk of the high-cancer strain. Milk, a fluid of fairly constant composition, offers obvious advantages for this work. Although ordinarily a colloidal suspension of casein and fat in a solution of proteins of lower molecular weight, the milk of the high-cancer strain also contains another protein, the virus.

Fractionation by a variety of physical and chemical methods is under way. Electrophoretic, ultracentrifugal, and electron microscopic evidence on the elimination of some components, and the isolation and concentration of other components was demonstrated.

EXCRETION OF STEROIDS IN THE FECES OF MICE OF VARIOUS STRAINS WITH AND WITHOUT THE MAMMARY TUMOR MILK AGENT. LEO T. SAMUELS (by invitation), JOHN J. BITTNER, and BARBARA K. SAMUELS (by invitation). (Department of Biochemistry, University of Utah Medical School, Salt Lake City, Utah, and Division of Cancer Biology, University of Minnesota, Minneapolis, Minn.)

The excretion of ketosteroids has been studied in various strains of mice with and without the mammary tumor milk agent. It appears that the absence of the milk agent is associated with increased fecal excretion of ketosteroids. Most of the ketosteroids excreted have been found in the unconjugated form. The possible significance of the difference was discussed.

COMPARATIVE STUDIES OF THE ESTROUS CYCLES IN RELATION TO THE MAMMARY TUMOR MILK AGENT. ROBERT A. HUSEBY and JOHN J. BITTNER. (Division of Cancer Biology, University of Minnesota, Minneapolis, Minn.)

Chemical analysis of the excreta of mice has shown that mice possessing the milk agent virus have a much lower 17-ketosteroid excretion than genetically similar

mice lacking the virus (Samuels and Bittner). As androgens inhibit the action of estrogens upon the vaginal mucosa as well as upon other organs, if the altered 17-ketosteroid excretion noted is due at least in part to a change in the production and/or metabolism of androgenetically active compounds, the estrous cycles of mice should vary according to the presence or absence of the agent. To test this the estrous cycles of groups of mice differing only in this one respect were compared. Strain A and C3H mice and their F_1 hybrids and hybrids between the dba and C3H strains were studied. It was found, generally, that mice possessing the agent showed vaginal cornification a greater percentage of the time than did those mice lacking the virus. Also the percentage of vaginal cornification of mice lacking the agent could be increased by foster-nursing such mice to females that possessed the agent. Mice of the C3H strain differed from the other groups studied, for in this strain there was no difference in the estrous cycles whether the animals possessed or lacked the agent. The reason for this is obscure at the present time.

EXPERIMENTAL ALTERATION OF THE CELLS OF A TRANSPLANTED TUMOR. C. W. HOOKER, C. A. PFEIFFER, and L. C. STRONG. (Department of Anatomy, Yale University School of Medicine, New Haven, Conn.)

A testicular tumor composed of primitive Leydig cells that arose spontaneously in a mouse of the Strong C strain has been carried through 24 serial subcutaneous transfers in untreated mice of the same strain. No change in the cytology of the tumor has been encountered. Occasionally the grafted tumor has invaded the muscle of the body wall, and in a few instances metastases to the kidneys, liver, and lungs have been recorded. In the last 20 transfers the graft has consistently attained a size of 2.5 by 1.5 cms. in 3 weeks. When grown in castrated males the cytology of the tumor remained unchanged and the condition of the genital system of the hosts indicated no production of androgen. When equine gonadotrophin was injected daily into castrated mice carrying the tumor, the tumor cells were transformed into morphologically mature Leydig cells, and the condition of the genital system indicated the secretion of levels of androgen approximating that of the normal male mouse. Thus an agent that will provoke cellular differentiation in the normal testis has also brought about full morphological differentiation and physiological activity in the cells of an apparently malignant tumor.

EFFECT OF ORCHIECTOMY ON ADVANCED CANCER OF THE BREAST IN MALES. IRA T. NATHANSON. (From the Medical Laboratories of the Huntington Memorial Hospital of Harvard University and the Tumor Clinic at the Massachusetts General Hospital, Boston, Mass., and Pondville Hospital, Massachusetts Department of Public Health, Walpole, Mass.)

Five males with advanced, primary, recurrent or metastatic cancer of the breast were subjected to bilateral orchiectomy and exhibited some form of beneficial response therefrom. These effects were seen primarily in the original lesion, lymph node and pulmonary metastases. Changes in osseous metastases were less well defined. All the patients exhibited a general improvement in their physical status. These effects are akin to those seen in patients with advanced cancer of the prostate gland following orchiectomy or estrogen therapy. In 2 patients estrogen therapy was instituted after recrudescence of the disease. No definitive effect was seen. The response is apparently only temporary, however, as 2 patients have succumbed, and the remaining patients have shown no evidence of reactivation of their disease 9 to 15 months following orchiectomy. These findings suggests further a hormonal control of certain type of neoplasms.

METABOLIC EFFECTS OF TREATMENT OF CARCINOMA OF THE PROSTATE. JOSEPH C. AUB, DOROTHY M. TIBBETTS, and IRA T. NATHANSON. (Massachusetts General Hospital, Boston, Mass.)

Over a period of several years, we have studied metabolic effects of castration and of stilbestrol in a few patients with carcinoma of the prostate. Changes in the excretion of calcium, phosphorus, nitrogen, and citrate were not dramatic as were the variations in the blood phosphatase levels. We are trying to analyze the influence of treatment upon the viability or function of tumor cells by determining the acid phosphatase in multiple biopsies of metastatic lymph nodes.

URINARY SEX STEROID BALANCE IN PROSTATIC DISEASE. WILLIAM T. SALTER, FRANCES D. HUMM, and JOHN B. GOETSCH. (Laboratories of Pharmacology and Toxicology and the Department of Surgery, Section on Urology, Yale University School of Medicine, New Haven, Conn.)

That hormones can influence the progress of prostatic cancer is well supported by clinical and laboratory evidence. Such work has led, in general, to the theory that the affected organism presents an environment in which androgenic elements are predominant. This has led logically to methods of therapy in current practice which are aimed at upsetting the prevailing hormone balance either by (a) removal of the testis or (b) supplying exogenous estrogen, or by using a combination of both methods.

By actual test, however, the urinary excretion in such cases indicates an imbalance in the opposite direction from that which has been assumed to exist. The ratio of estrogen (in μgm.) to "androgen" (17-ketosteroids in mgm.) as determined microchemically, is strongly in favor of estrogens in a high percentage of cases of prostatic disease. The E/A ratio is under 1.0 in healthy young adult males, while ranging from 2.0 to 10 in ovulating adult females. In contrast, males with prostatic hypertrophy or prostatic carcinoma frequently show ratios in the female range, and occasionally above 10. This paradoxical trend of the steroid ratio bears no relationship to the degree of malignancy involved. It does fur-

nish evidence which indicates that the relation of androgens and estrogens to prostatic disease must be reevaluated.

THE RELATIONSHIP OF THE NUCLEOLUS TO CYTOPLASMIC NUCLEIC ACIDS AND PROTEINS IN DIFFERENT CONDITIONS OF GROWTH IN RAT LIVER. ROBERT E. STOWELL. (Department of Pathology, Washington University School of Medicine, St. Louis, Mo., and Institute for Cell Research, Karolinska Institute, Stockholm, Sweden)

Rats were kept on a protein-free diet for periods up to 3 months to deplete the protein of the body. A few protein-depleted animals were then placed on a high protein diet for intervals up to 8 days. The liver of numerous normal controls, of 6 protein-depleted and 3 partially protein-repleted rats were frozen-dried or fixed in Stieve or Carnoy fluid. Some sections were stained with hematoxylin and eosin and others by the Feulgen reactions for thymonucleic acid. Fixed and unfixed sections were photographed with ultraviolet light of 2,570 A.

The nucleoli of the hepatic cells of rats on a protein-free diet increased to twice their normal size and the nuclei and cytoplasm decreased in volume. After a few days on a high protein diet the size of the nucleoli decreased and their number per nuclear section increased. The changes in the cytoplasmic absorption at 2,570 A were suggestive of an increased nucleotide content. The results of these preliminary experiments, when compared with similar experiments on liver cells in regeneration and in neoplastic transformation, show that there are large morphologic variations in the nucleolus of hepatic cells under different conditions of growth.

GROWTH RATE OF TRANSPLANTED TUMORS IN RELATION TO LATENT PERIOD AND HOST VASCULAR REACTION. GLENN H. ALGIRE, and FRANCES LEGALLAIS. (National Cancer Institute, Bethesda, Md.)

Transplanted tumors that have been studied in transparent chambers inserted into mice fall into two general classes in respect to their growth rate and vascular development. Among the rapidly growing group studied so far are included sarcomas, mammary gland carcinomas, and a malignant epithelial tumor of the skin. These elicited new capillary sprouts from the host as early as 2 to 3 days, the surrounding host vessels became hyperemic and numerous leukocytes accumulated about the implants. The percentage of the vascular tissue rose to approximately 50 per cent then stabilized at that level. The capillaries of the tumors mentioned above had an average diameter 5 times greater than those in a normal tissue (striated muscle), appearing as enormous sinusoid-like vessels which showed little tendency to differentiate into arterioles and venules.

In striking contrast to the rapidly growing tumors that killed the host in from 3 to 6 weeks, were the slowly growing tumors which killed the host in from 3 to 6 months. These included the Harding-Passey and Cloudman S91 pigmented melanomas, and an amelanotic melanoma derived from the S91. The slow growth rate of these tumors was correlated with a prolonged latent period prior to capillary proliferation, usually 8 days or more. In addition, vascular levels in these tumors rarely exceeded that of the vessels in the surrounding subcutaneous connective tissue, and were less than one half that of the rapidly growing tumors. There was very little leukocytic accumulation about the implants and vascular hyperemia in the surrounding tissues was lacking. The capillaries formed were small in diameter, like those of normal striated muscle, and showed considerable differentiation into arterioles and venules.

METABOLIC CHARACTERIZATION OF TRANSPLANTED MOUSE MELANOMAS BY HIGH OXIDATIVE RESPONSE TO PARAPHENYLENEDIAMINE. MARIE L. HESSELBACH, DEAN BURK, GLENN H. ALGIRE, CLARA FISCHER, and FRANCES Y. LEGALLAIS. (National Cancer Institute, Bethesda, Md.)

Tissue slices of the 3 transplantable mouse melanomas, the Harding-Passey, the Cloudman S91 pigmented melanomas and the S91A amelanotic melanoma, showed a metabolism consistent with that of malignant tumors generally, in regard to aerobic and anaerobic glycolysis, oxygen consumption, respiratory quotient, and other related derived quotients.

On the other hand, all 3 melanomas showed a much greater percentage stimulation (400 to 1,000) of oxygen consumption by paraphenylenediamine than any other tumors tested to date (0 to 150 per cent), and the stimulation was in all cases essentially eliminated by cyanide. This greatly enhanced stimulation of oxygen consumption by paraphenylenediamine offers the possibility of a biochemically new characterization and mode of diagnosis of melanomas, amelanotic as well as pigmented, that is readily subject to further testing with a variety of other melanomas.

The marked stimulation of oxygen consumption caused by paraphenylenediamine in cyanide-free tissue slices of the melanomas, as compared with other tumors tested, may be interpreted as indicating that the ratio oxidized/reduced cytochrome c is considerably higher in these melanomas than in other tumors, and, in fact, in the range in normal and embryonic tissues generally. This is indicative of a relatively high level of oxidation-reduction potential within the melanoma cells, either intracellularly throughout or locally in certain cell areas.

THE EFFECT OF AGE ON REGENERATION OF RAT LIVER FOLLOWING PARTIAL HEPATECTOMY. NANCY L. R. BUCHER, ANDRÉ GLINOS, and JOSEPH C. AUB. (Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Mass.)

It seems possible that the frequent association between cancer and old age may express some significant aspect of the genesis of cancer. Accordingly it seems important to formulate the response of ageing tissues to growth stimuli.

Regenerating rat liver has been chosen for such a study because its restorative capacity can be accurately quantitated. Previous investigators have found that age delays mitosis in this tissue, and that it retards restoration of liver.

In the present experiment rats of accurately known ages were divided into young (4 to 6 weeks), adult (6 to 8 months) and old (1¾ to 2½ years) animals. Many of the latter group showed the characteristics of senility. The main lobes of the liver, constituting approximately 68.4% of the total, were removed, and the method of Brues, Drury and Brues (*Arch. Path.*, 22-658, 1936) followed in determining the percentage of restoration in terms of (1) mass and (2) number of cells. Rats were autopsied at intervals of 16 and 30 hours, and 3, 7 and 14 days after operation.

The young rats, in whom regeneration was superimposed on active growth, were not strictly comparable to the other two groups. In general the regenerative capacity decreased with age. Restoration of liver mass was retarded in the adult and old rats as compared with the young rats, and restoration of hepatic cells to their original number was retarded in the old rats as compared with the other two groups.

THE CITRIC ACID CONTENT OF TUMOR TISSUE AND OF TUMOR-BEARING ANIMALS. FRANCES L. HAVEN, and CHALLISS RANDALL. (Department of Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

The citric acid content of Walker 256 and of liver, blood, and kidneys of rats bearing this tumor has been determined. The necrotic portion of 21 tumors contained 4 to 20 times more citric acid than the non-necrotic portion. The blood of tumor-bearing animals was normal in citric acid. The kidneys and, to a lesser extent, the livers of rats bearing this tumor were higher in citric acid than similar organs of rats without tumors.

ON DEFECTIVE PLASMA PROTEIN FORMATION IN PATIENTS WITH GASTRIC CANCER. F. HOMBURGER, AURELIA POTOR, and N. F. YOUNG. (Sloan-Kettering Institute for Cancer Research, New York, N. Y.)

Intractable hypoproteinemia is part of the systemic disease found in patients with gastric cancer. This study of nitrogen balance and plasma protein regeneration was made on 13 patients with gastric ulcers, operable and inoperable cancer of the stomach. It was found that on high protein intakes, sufficient to produce plasma protein regeneration in patients with gastrectomies for ulcers, plasma protein levels of patients with gastric cancer remained low or continued to fall. The increase of circulating plasma protein in 2 cases was due to globulins. This finding, together with the fact that even in the presence of positive nitrogen balance for as long as 80 days no increase of circulating plasma protein occurred, suggests an anomaly of protein metabolism in these patients.

HISTOCHEMICAL PHOSPHATASE REACTION IN MOUSE SARCOMAS CR 180 AND 37 FOLLOWING ADMINISTRATION OF BACTERIAL POLYSACCHARIDE. MORRIS BELKIN, and ELMER D. BUEKER (by invitation). (Departments of Pharmacology and of Anatomy, Medical College of South Carolina, Charleston, S. C.)

Swiss albino mice carrying 2 weeks old implants of sarcoma CR 180, and dba mice carrying similar implants of sarcoma 37 were each given 0.1 mgm. of bacterial polysaccharide intraperitoneally. They were then sacrificed, as were control animals, in groups of 2 or 3, at half-hourly or hourly intervals for the first 4 hours, and at 8, 12 and 24 hours after injection. The tumor tissue was fixed in chilled acetone for acid phosphatase, and in 80 per cent alcohol for alkaline phosphatase preparations. Gomori's histochemical method was used for both acid and alkaline phosphatase reactions, with minor modifications.

For both acid and alkaline phosphatases, 5 different substrates were used, with varying incubation periods as follows:

	Acid pH	Alkaline pH
Glycerophosphate	6½	8
Adenylic acid	7?	8
Fructose diphosphate	7?	?
Lecithin	7?	7?
Yeast nucleic acid	8?	8

No striking effects were obtained for any of these substrates, for either acid or alkaline phosphatase reaction.

The tumors incubated with glycerophosphate and yeast nucleic acid on the acid side, and all the substrates on the alkaline side, showed a marked increase in staining potency in 2 to 3 hours after polysaccharide administration.

Microscopically, at that interval, the nuclear walls and nucleoli were somewhat more darkly stained. But, as the cytotoxic action of the polysaccharide continued, with progressive dissolution of nuclear contents into granular fragments, and their dispersal into the cytoplasm, the intensity of the staining diminished.

It is concluded that the cytotoxic effect of bacterial polysaccharide is not mediated through attenuation or destruction of the phosphatase enzymes, in so far as it has been studied by this particular histochemical technique.

REDUCTION IN TOXICITY OF *SERRATIA MARCESCENS* POLYSACCHARIDE TO TUMOR-BEARING MICE PRODUCED BY UPJOHN CO. BEEF ADRENAL EXTRACT. LYLE BECK, IRENE DILLER, BERTINA BLAUCH, and MARY FISHER. (Lankenau Research Institute, Philadelphia, Pa.)

The *Serratia marcescens* tumor necrotizing polysaccharide isolated by Shear and his coworkers (*J. Nat. Cancer Inst.*,) produces toxic effects which may culminate in death when the mice bear large tumors or when a dose many times that required to produce extensive necrosis is given to mice bearing small tumors.

Five hundred micrograms of *S. marcescens* polysaccharide preparation P. of the National Cancer Institute

caused death within 48 hours in 64 of 75 mice bearing 7 day tumors (sarcoma 37). Most of these mice died within a few hours after intraperitoneal injection of the polysaccharide. Another group of 45 mice bearing 7 day tumors was given 0.25 cc. of Upjohn Co. beef adrenal extract, 500 μgm. of P$_s$ polysaccharide and another 0.25 cc. of adrenal extract at the end of the working day. Of these mice, 22 survived 48 hours or longer. The probability of this difference in survival being due to chance was calculated using Chi Square and was found to be about 1 in 10,000.

On the other hand, no evidence was secured that Upjohn Co. concentrated hog adrenal extract in oil is effective in counteracting the lethal effects of 500 μgm. of polysaccharide preparation P$_s$ given to mice bearing 7 day tumors.

STUDIES OF PULMONARY TUMOR INDUCTION IN MICE BY DERIVATIVES OF CARBAMIC ACID. C. D. LARSEN. (National Cancer Institute, Bethesda, Md.)

Examination of the phenomenon of pulmonary tumor induction in mice by ethyl carbamate and other derivatives of carbamic acid have been extended. In strain A mice single injections of a narcotizing dose of ethyl carbamate initiated increases in incidence and frequency of lung tumors. Although an initial response was noted after 1 month, maximum effects were not observed until 5 months had elapsed.

Ethyl carbamate (urethane) was relatively specific in its capacity to induce lung tumors. Studies of ester homologues of urethane, other than those previously reported, substantiate the specificity of urethane. β-chloroethyl carbamate and trichloroethyl carbamate were inactive; propyl carbamate and isopropyl carbamate exhibited about 1 and 5 per cent, respectively, of the activity of the ethyl ester. N-alkylated ethyl carbamates, with one exception, tended to decline in activity as the extent of alkylation was increased. Mono-N- and di-N-methyl ethyl carbamates exhibited only about 10 and 5 per cent, respectively, of the activity of urethane. Mono-N-isopropyl ethyl carbamate, however, elicited a striking increase in lung tumors; an activity approaching 50 per cent of that of urethane was noted.

Embryonic lung tissue was found susceptible to the oncogenic action of urethane. Litters from pregnant mice that had been injected with a single narcotizing dose of urethane prior to parturition were kept until 6 months of age. Striking increases in the incidence and multiplicity of lung tumors in the offspring were observed. Essentially identical results followed either intraperitoneal or intravenous injection of the pregnant mice. The response of pulmonary tissue to *in utero* exposure to the agent tended to vary inversely with the injection-parturition interval.

AN EXPERIMENTAL STUDY OF SINGLE TRAUMA MALIGNANCY. WILLIAM L. SIMPSON. (The Barnard Free Skin and Cancer Hospital, and Washington University School of Medicine, St. Louis, Mo.)

The skin of the Swiss strain mouse is rendered unusually susceptible to the action of carcinogenic agents by the prolonged application to it of carcinogenically inactive solutions of methylcholanthrene in anhydrous lanolin. Unless actively carcinogenic compounds are applied to the skins of these "sensitized" mice, they usually remain free from cancer until death, and neither structurally nor chemically do their skins resemble those in which precancerous changes have been initiated by "active" solutions of methylcholanthrene in benzene.

Experiments have been conducted on such hypersusceptible skin, as well as on normal mouse skin, to test the cancer-evoking potentialities of a single severe trauma. Three types of injury were inflicted: (1) burning with a hot glass rod, (2) crushing of the skin with pliers, and (3) exposure to a massive localized dose of roentgen irradiation.

Only rarely does the normal mouse respond to a solitary trauma by the development of a malignant tumor, a result in agreement with most earlier observations. In sharp contrast are the results on "sensitized" mice. Malignant tumors did not follow injuries by crushing or by x-ray "burns," but in two groups of mice subjected to trauma by burning with a hot glass rod malignant tumors appeared in 42 per cent and 65 per cent respectively. Carcinomas, sarcomas and carcinosarcomas were produced. In both groups approximately 80 per cent of the tumors arose *at the site of the preceding injury*. The average period of induction, dated from the time of injury, was 7 months.

The bearing of these experiments on the much discussed problem of single trauma cancer in man was considered.

DIFFUSIBLE AND NON-DIFFUSIBLE CALCIUM IN NORMAL AND METHYLCHOLANTHRENE-TREATED MOUSE EPIDERMIS. A. I. LANSING and M. H. AU. (Department of Anatomy, Washington University School of Medicine, and Barnard Free Skin and Cancer Hospital, St. Louis, Mo.)

Carruthers and Suntzeff in 1943 established that epidermal calcium is significantly decreased in methylcholanthrene-induced hyperplasia and carcinoma. The present investigation was designed to explore further this pronounced shift in total calcium and to determine whether the ratio between free and bound calcium in early and late hyperplasia and carcinoma is altered.

The method employed for separation of free and bound calcium (measured as diffusible and non-diffusible calcium) was based upon the ultrafiltration technic of Mazia in 1937, and calcium was determined by the method of Lindner and Kirk, the same year.

The ratio of diffusible to non-diffusible calcium in normal 3 month old Swiss mice was 1:1.6; early (20 days) hyperplasic epidermis revealed no significant alteration of this ratio but confirmed the 50 per cent drop in total calcium reported by Carruthers and Suntzeff. Study is being made of the diffusible and non-diffusible calcium ratio in late hyperplasia (60 days) and carcinoma.

HYALURONIDASE AND THE GROWTH OF MA-
LIGNANT EPITHELIAL TUMORS. A. R.
GOPAL-AYENGAR, and WILLIAM L. SIMP-
SON. (The Barnard Free Skin and Cancer Hospital,
and Washington University School of Medicine,
St. Louis, Mo.)

Although an association between "spreading factors"
and the growth of malignant tumors has been recognized
for some years, the nature of the relationship has never
been elucidated. We have now tested by direct experi-
ments the hypotheses formulated in 1943 by Cramer and
Simpson on a possible mechanism for this association.
Included in the investigations were: (1) the effects of a
spreading factor from testis (hyaluronidase) on the
growth and invasive capacities of a mouse-transplantable
squamous cell carcinoma, (2) the relation of hyaluroni-
dase and anti-hyaluronidase antibodies to the develop-
ment of the transplantable tumor, and (3) the effect of
the enzyme on carcinogenesis in response to methyl-
cholanthrene.

Local injection of the hyaluronidase about the base of
established cancer transplants resulted in the enhance-
ment of invasive growth with a striking destruction of
muscle and bone by the tumor. In a few instances the
local injection was followed promptly by the appearance
of distant metastatic lesions.

Results of the other experiments, which were not then
quite completed, were described at the meeting.

THE ROLE OF SEBACEOUS GLANDS AND HAIR
FOLLICLES IN EPIDERMAL CARCINOGENE-
SIS IN MICE. V. SUNTZEFF, C. CARRUTHERS,
and E. V. COWDRY. (From the Barnard Free
Skin and Cancer Hospital, and the Department of
Anatomy, Washington University School of Medi-
cine, St. Louis, Mo.)

Previous studies in this laboratory revealed that young
New Buffalo mice developed squamous cell carcinoma
more rapidly and in a higher percentage than did old mice
of the same strain after the topical application of methyl-
cholanthrene. This difference led to an investigation of
the response of the skin of very young mice (2 to 10 hours
after birth) to a single application of the same carcinogen.
Thirty mice were treated in this fashion, and 19 months
after the application of the carcinogen, 23 mice were alive
without evidence of tumor formation. A possible
morphological basis for this lack of responsiveness was
found in a detailed study of the development of the skin
and its associated structures from the time of birth until
the skin was completely developed. The hair follicles
and sebaceous glands were found to be rudimentary at
the time the carcinogen was applied, and the epidermis
was well differentiated and covered with a thick layer of
keratin. The failure of very young mice to develop cancer
may be due to the following factors: Inability of the
carcinogen to penetrate through the thick epidermis or to
reach the few rudimentary sebaceous glands via the hair
follicles, only a few of which have hair reaching the ex-
terior. That the hair follicles and sebaceous glands play
an important role in epidermal carcinogenesis in mice is
quite apparent from this study.

STUDIES ON THE TRANSMISSION OF AVIAN
VISCERAL LYMPHOMATOSIS. I. VARIATION
IN TRANSMISSIBILITY OF NATURALLY
OCCURRING CASES. BURMESTER, B. R.,
and DENINGTON, E. S. (U. S. Regional Poultry
Research Laboratory, East Lansing, Mich.)

The transmissibility of tumors from 10 cases of natural-
ly occurring visceral lymphomatosis was tested by in-
oculation of cellular and cell-free preparations into groups
of 14 to 21 chicks 1 day of age. The recipient chicks were
relatively free from prior infection since none of 41 non-
inoculated controls developed tumors during an experi-
mental period of 183 days.

Lymphomatous tumors of the viscera were reproduced
(an incidence of 14 to 85 per cent in 93 to 183 days) in
recipients of cell-containing preparations from 8 of the
original tumors. Similar tumors were produced (an in-
cidence of 39 to 91 per cent in 183 days) by cell-free
preparations from 5 of the original tumors. In addition
to the visceral tumors, preparations from 1 tumor also
produced a high incidence of osteopetrosis.

Of the 10 donors that supplied visceral tumors, 7 also
had gross or microscopic evidence of neurolymphomatosis.
Gross neural lesions appeared in 1 to 4 chickens of several
groups, however, there appeared to be no direct relation
between the presence of this lesion in the donor and the
number of recipients that developed neural or visceral
lymphomatosis.

Tumors of some, but not all, cases of visceral lympho-
matosis are transplantable, and part of these tumors may
be transmitted to chicks by inoculation with filtrates.
The active agent or agents are of a size which will allow
them to pass readily through bacteria-retaining filters.

TRANSPLANTATION OF THE ROUS CHICKEN
SARCOMA INTO THE ANTERIOR CHAMBER
OF THE MOUSE EYE. EDWARD W. SHRIG-
LEY. (From the Department of Bacteriology and
Immunology, Yale University School of Medicine,
New Haven, Connecticut)

The Rous chicken sarcoma placed into the eye of the
mouse grows to fill the chamber and frequently herniates
to the exterior through the cornea. The growth behavior
of the sarcoma in the mouse eye is similar to that in the
eye of the guinea pig. However, in the former the tissue
persists longer before undergoing regression. Transplants
capable of producing growths in chicks have not been
obtained from mice after 15 days of residence. Chicks
injected directly with this mouse growth may show, in
addition to the local tumor, hemorrhagic disease and
periosteal sarcomas. Subsequent passages in chicks in-
dicate that unlike the guinea pig passage agent, the virus
has not undergone alteration in specificities nor has it
increased in potency. On the contrary, data suggest that
the mouse passage virus has lost some of its virulence
while its tissue specificities are no different from those of
the stock Rous agent.

THE MORPHOLOGIC STABILITY OF SIX
STRAINS OF MALIGNANT MOUSE FIBRO-

BLASTS GROWING *IN VITRO*. WILTON R. EARLE. (National Cancer Institute, Bethesda, Md.)

The production of six strains of sarcoma cells from one parent strain of mouse fibroblast growing in an entirely heterologous medium *in vitro* has been previously reported. Of these six, strains H, J, L, N and O had been treated with a concentration of 1 μgm. of 20-methylcholanthrene per ml. of culture media for 6, 32, 111, 184, and 406 days respectively. The degree of morphologic alteration in these cell strains was apparently directly associated with the time the cell strains had been subjected to the carcinogen. Strain D, the presumably untreated control strain, also underwent a very limited morphologic alteration, but never showed as great a change as the cells of strain H, which were subjected to the carcinogen for 6 days.

The last of these cell strains was removed from 20-methylcholanthrene on September 16, 1942, and since that time all strains have been grown in the same heterologous culture medium of chicken plasma, horse serum, and chick embryo extract, and under the same experimental culture conditions.

Periodic photographs of these living cultures from December 17, 1942, through December 19, 1946, showed that strains J, N, and O have undergone certain limited secondary alterations within this interval. Strains D, H, and L, however, have shown no recognizable change in their respective characteristic induced morphologies since December 17, 1942. Allowing generously 5 days for each intermitotic interval in these three cell strains, it seems that the respective characteristic induced morphologies of these three cell strains have been stable for over 290 consecutive cell generations.

THE USE OF PURIFIED FIBRINOGEN WITH CERTAIN STRAINS OF NORMAL AND MALIGNANT FIBROBLASTS IN TISSUE CULTURES. VIRGINIA J. EVANS, HELEN M. DYER, and MARGARET G. KELLY. (National Cancer Institute, Bethesda, Md.)

An attempt was made to obtain a more chemically reproducible solid culture medium for tissue culture metabolic studies than has been possible by the use of plasma. A study has been made of bovine fibrinogen prepared by a number of different procedures. Test cell strains used have all been subcutaneous mouse fibroblasts and have included 3 strains of presumably normal mouse fibroblasts, one freshly explanted *in vitro* and two grown *in vitro* for more than 3 years. Earle's sarcoma strains D, H, J, L, N, and O were also used. All cultures were grown in Carrel D3.5 flasks and the supernatant culture medium has been 40 per cent saline, 40 per cent horse serum and 20 per cent chick embryo extract.

Results to date indicate that different cell strains vary substantially in their tendency to lyse this solid substrate. Of the cell strains tried, strain L, alone was unable to lyse the clot to any perceptible degree. All three strains of normal cells showed rapid lysis of the clot as did the sarcoma strains D, H, J, N, and O.

HEREDITARY EOSINOPHILE LEVELS IN THE ACQUIRED RESISTANCE OF THE RABBIT TO THE BROWN-PEARCE TUMOR. ALBERT E. CASEY and GEORGE R. DRYSDALE. (Department of Pathology, The Baptist Hospital, Birmingham, and the Holy Name of Jesus and Baptist Memorial Hospitals, Gadsden, Ala.)

Previous studies by our group demonstrated hereditary variations in the blood eosinophile levels of normal rabbits but none for the neutrophiles or monocytes. High pretransplantation eosinophile levels were associated with a lower incidence and number of metastases, and a lower mortality in animals receiving successful transplants than low pretransplantation levels. No such relation for the neutrophile or monocyte levels could be demonstrated.

Because the eosinophile effect did not seem to become manifest until the seventh week after inoculation 98 additional animals were studied, giving a cumulative total of 283 young adult male rabbits received from breeders. Of these, 159 had the blood level of each of nine blood cell factors within normal limits for the species, and were seemingly free from intercurrent disease. These 159 normal animals were inoculated intratesticularly with the Brown-Pearce tumor, and surviving animals were sacrificed two months thereafter.

The cumulative data indicate that the pretransplantation eosinophile level bears no apparent relationship to the course of the Brown-Pearce tumor during the first six weeks after inoculation. Its effect appears in the seventh week and persists with the characteristic and statistically significant pattern described above. It especially seems to affect the incidence of hematogenous metastases.

The seventh week corresponds to the beginning of regression or the turning point of this neoplastic disease as first described by Brown and Pearce and later by Malluche. Thus a relationship between the hereditary eosinophile level and the acquired resistance of the rabbit to the tumor is indicated.

RETARDATION OF GROWTH AND METABOLISM OF NORMAL AND MALIGNANT CELLS DURING CONTINUOUS CULTURE. JOHN H. HANKS (by invitation), GEORGE O. GEY, and RACHEL BARRETT (by invitation). (Division of Cell Physiology, Department of Surgery, Johns Hopkins Hospital and Medical School, Baltimore, Md., and Leonard Wood Memorial Department of Bacteriology, Harvard Medical School, Boston, Mass.)

Throughout the life of multicellular organisms, most of the tissues and organs are capable of carrying on their specific function with a fairly stable cell population and, therefore, at a low maintenance rate of growth. When cells are released from the organization and control of the host and are explanted in tissue cultures, conditions are usually provided which cause them to migrate and divide rapidly. Since a major portion of biological and medical interest in the results of tissue cultivation depend

on interpretation in terms of post-embryonic or adult physiology and pathology, it is obvious that the art and science of tissue cultivation need some reorientation in the direction of maintaining more stable populations of cells without rapid multiplication. Maintaining cells at metabolic levels approximating those of postpartum physiology is of value in studying problems concerned with cytology, nutrition, endocrine secretion, antibody formation, the interrelations of cells and infectious agents, and the riddle of differentiation and malignancy. By lowering cell metabolism through reduction in temperatures of maintenance and by decrease in concentration of nutrients, it has been possible to perpetuate strains of normal and malignant cells over long periods of time with minimal effort. Reduced temperature levels thus far investigated include 28°, 31°, and 34° C. The results reported include studies on normal human and rat fibroblasts and several strains of rat sarcoma. The effects of lowering temperature and nutrient supply upon rate of growth, duration of mitosis, cultural behavior, and cytology were discussed.

FURTHER OBSERVATIONS ON THE CONVERSION OF NORMAL INTO MALIGNANT CELLS *IN VITRO*. GEORGE O. GEY, and MARGARET K. GEY (by invitation). (Division of Cell Physiology, Department of Surgery, Johns Hopkins Hospital and Medical School, Baltimore, Md.)

This study is concerned with a series of permanent alterations occurring in continuous cultures of normal rat mesenchyme cells and leading to the production of malignant cells. The strains studied include normal, altered normal, and malignant cell strains of *autologous* origin which have been under cultivation for eight and one-half years. It has been possible to make direct comparison between a normal and a malignant strain derived from it. The data to the present time implicate factors contributed by a culture medium totally heterologous to the strains studied. No known extraneous carcinogenic agents have been found to play a part in these conversions which occurred in stocks of normal cell strains. Differences between normal and malignant autologous strains were discussed.

IS AEROBIC GLYCOLYSIS OF AN INTENSITY CHARACTERISTIC OF CANCER TISSUE A NORMAL METABOLIC FEATURE OF THE MUCOSA OF THE SMALL INTESTINE? OTTO ROSENTHAL. (Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pa.)

In 1941 Dickens and Weil-Malherbe reported that the rate of aerobic glycolysis of normal duodenal or jejunal mucosa of rat and mouse equals that generally obtained with cancer tissue. These authors believed they had eliminated the possibility of an artefact in spite of the exceptional observation that the aerobic glycolysis was as high as the anaerobic glycolysis. Such complete absence of the Pasteur effect has never before been observed in undamaged tissues whether normal or malignant.

Since the mucous membranes of murine species are extremely fragile the metabolism of the more stable duodenal mucosa of the rabbit was studied manometrically by means of Warburg's indirect method. In addition, lactic acid was determined colorimetrically with the method of Barker and Summerson.

The rates of respiration and of anaerobic glycolysis of the rabbit mucosa approximated those obtained with duodenal mucosa of the rat by Dickens and Weil-Malherbe. Q_{O_2} and $Q_G^{N_2}$ averaged 9.5 and 7.9 respectively (initial dry weight basis, 60 minutes). The aerobic glycolysis, however, amounted to but 10 per cent of the anaerobic glycolysis. The Pasteur effect was thus evident. Persistence of a small aerobic glycolysis is commonly found with normal tissues *in vitro*.

While these results do not eliminate the possibility that the high aerobic glycolysis of murine mucosa of the small intestine is a peculiarity of the species, the known absence of species differences in the metabolism of colonic mucosa does not favor this interpretation, but rather suggests an artefact.

MICROMETRIC INVESTIGATIONS ON MYELOMA CELLS AND NORMAL BONE MARROW PLASMA CELLS. HARALD GORMSEN. (Department of Pathology, University Institute of Forensic Medicine, Copenhagen, Denmark)

Micrometric investigations by ocular micrometer have been carried out on normal bone marrow plasma cells (smears of sternal punctures and sections of bone marrow from 15 normal adult persons) and on myeloma cells (smears of sternal punctures and sections of myeloma tissue from 29 patients). In each preparation 50 cells and their nuclei have been measured in longitudinal and transverse direction. The average values of the 50 measurements have been subjected to statistical analysis.

In 18 of the 29 cases of myeloma both the nuclei and total cell size were significantly larger than normal plasma cells in bone marrow. In 8 cases, only the nuclei of the myeloma cells were significantly larger than nuclei of normal plasma cells in the bone marrow, whereas 3 myeloma cases showed cell- and nuclei-sizes that did not differ from normal bone marrow plasma cells.

Consequently, in the majority of myeloma cases (in the present material 26 out of 29) the myeloma cells differ unmistakably from normal bone marrow cells. In a few cases (in the present material 3 out of 29) myeloma cells in all aspects (cell size, nucleus size, nuclear structure, etc.) are morphologically identical with normal bone marrow plasma cells.

This observation is of practical importance in the use of sternal punctures for the differential diagnosis between myelomatosis and conditions with reactive plasma cell proliferation in the bone marrow (infections, etc.).

No significant relation could be demonstrated between the degree of morphological abnormality of myeloma cells and the clinical symptoms or the course of the myeloma cases.

THE EFFECT OF SOME CARCINOGENIC AMINOAZO DYES ON THE AUTOXIDATION OF LINOLEIC ACID. H. P. RUSCH, and J. A. MILLER. (McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.)

Previous publications from this laboratory have demonstrated that carcinogens including certain azo dyes inhibit the autoxidation of unsaturated lipids. The present paper describes in more detail the effect of *p*-dimethylaminoazobenzene and its demethylated derivatives on the autoxidation of purified linoleic acid. The aminoazo dyes were purified by chromatographic adsorption. Known quantities of linoleic acid and the azo dyes were placed in Warburg flasks and the rate of autoxidation was followed manometrically at 36.5° C. The flasks contained linoleic acid alone or with varying levels of *p*-dimethylaminoazobenzene (DAB), *p*-monomethylaminoazobenzene (MAB), or *p*-aminoazobenzene (AB).

DAB and MAB both increased the latent period of oxidation of linoleic acid, the former being a more effective antioxidant than the latter, and the antioxidant effect of each dye was proportional to the concentration employed. Thus, when DAB was used at concentrations of M/200, M/100, and M/50 the oxidation of the linoleic acid at the end of the first 24 hour period had progressed only 56, 29, and 0 per cent respectively as compared to the acid alone. With the same levels of MAB, the amount of oxidation was 73, 45, and 35 per cent respectively. Contrary to the inhibiting effect of the methylated dyes, AB shortened the latent period slightly.

As the autoxidation proceeded, the azo dyes disappeared from the flasks and DAB and MAB were found to be demethylated. At the end of 30 hours 90 per cent of the DAB initially added had disappeared from the flask but MAB appeared in amounts equal to 85 per cent of the starting level of DAB. Small amounts of AB were also found throughout the run. MAB disappeared more slowly than DAB during the course of the oxidation and it was found to be demethylated to AB. AB disappeared very rapidly in oxidizing linoleic acid and no other basic dye was detected in the mixture.

THE INHIBITION OF THE GROWTH OF *LACTOBACILLUS CASEI* BY *P*-MONOMETHYLAMINOAZOBENZENE AND ITS REVERSAL BY RIBOFLAVIN. E. C. MILLER, H. N. KINGSLEY, and J. A. MILLER. (McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.)

The activity of the hepatic carcinogens *p*-dimethylaminoazobenzene and *p*-monomethylaminoazobenzene can be greatly modified by the character of the diet in which they are fed. In particular, feeding diets high in riboflavin to rats greatly delays tumor development due to these compounds. This antagonism has now been studied using the growth of *L. casei* as the end point; the growth of this organism is proportional to the riboflavin content of the medium. The bacteria were grown in the medium of Roberts and Snell except that in certain cases amino acids were substituted for a part of the hydrolyzed casein. Growth was measured turbidimetrically 24 hours after inoculation. *p*-Monomethylaminoazobenzene was generally used because it is 10 times as soluble as *p*-dimethylaminoazobenzene in aqueous media.

When 1 to 3 μgm. of *p*-monomethylaminoazobenzene were added per ml. of medium, growth was inhibited by 60 to 90 per cent at riboflavin levels of 0.01 to 0.15 μgm. per ml. Increasing the level of riboflavin to 1.25 μgm. per ml. decreased the inhibition to 0 to 40 per cent; higher levels of riboflavin were impractical because of the poor solubility of the vitamin. The inhibition due to the dye could also be reversed by an unidentified constituent present in fresh pancreatic digests of casein; the activity of this factor decreased on storage in the cold for 4 to 8 weeks. When *Saccharomyces cerevisiae* was grown anaerobically in the same medium, its growth was inhibited 20 to 40 per cent at levels up to 0.2 μgm. of riboflavin per ml. Larger amounts of riboflavin usually reduced the inhibition to 10 per cent or less. *S. cerevisiae* destroyed 80 to 90 per cent of the *p*-monomethylaminoazobenzene in the medium at the high levels of riboflavin while *L. casei* destroyed only 10 to 20 per cent.

SUSCEPTIBILITY OF STRAIN C MICE TO *o*-AMINOAZOTOLUENE. H. B. ANDERVONT, and THELMA B. DUNN. (National Cancer Institute, Bethesda, Md.)

Female mice of strain C are much more susceptible than males to hepatic lesions induced by *o*-aminoazotoluene. Castration of males considerably increases their susceptibility while castration of females lowers their susceptibility. Administration of testosterone propionate to castrated males or females lowers their susceptibility to that of intact males. The compound induces hemangioendotheliomas in both sexes. The site of origin of these tumors is influenced by the site of administration of the compound.

TUMORS PRODUCED IN RATS AFTER INGESTION OR PAINTING OF 2-NITRO, 2-AMINO, *N*-ACETYL-2-AMINO, AND *N*-DIACETYL-2-AMINO FLUORENE. H. P. MORRIS, C. S. DUBNIK, T. B. DUNN, and J. M. JOHNSON. (National Cancer Institute, Bethesda, Md.)

The carcinogenic effect on the rat of 4 derivatives of fluorene were studied after both ingestion and painting. In the feeding experiments each derivative was fed to rats at a level of 0.05 per cent in a low-fat synthetic diet for 160 days. The average daily ingestion of carcinogen ranged from 4.0 to 4.7 mgm. In the painting experiments a 2 per cent acetone solution of each compound was applied thrice weekly to the scapular region. The estimated amount of carcinogen given with each application was 0.5 mgm. during the first 6 months and 1.00 mgm. thereafter. The painted rats were fed a stock diet. Autopsies were made after the appearance of tumors or

when death appeared imminent. Sixty-four tumors were identified histologically in 104 treated animals. No tumors were found in control animals.

Distant tumors were produced by all 4 derivatives after either ingestion or painting. 2-Aminofluorene was the only compound producing skin tumors. The majority of liver tumors were observed in rats after ingesting either the mono or diacetyl derivative. The 2-nitro derivative induced no liver tumors. The results of these experiments suggest for both types of administration an increasing order of carcinogenicity from the nitro to the amino to the mono or diacetyl derivative. The type of distant tumors produced, while not dependent on the route of administration, seems to be influenced by it.

PARALLEL EFFECTS OF CERTAIN DIETS UPON THE RETENTION OF RIBOFLAVIN AND THE FORMATION OF HEPATIC TUMORS IN THE LIVERS OF RATS. A. C. GRIFFIN, and C. A. BAUMANN. (Department of Biochemistry, University of Wisconsin, Madison, Wis.)

When p-dimethylaminoazobenzene was fed to rats, the amount of riboflavin in the liver varied with the concentration of this vitamin in the diet: liver storage was lower on synthetic or semi-synthetic diets containing 0.7γ of riboflavin per gm. of diet than on similar diets containing 2.0γ of riboflavin per gm. The rate of tumor formation was faster on the lower level of riboflavin intake, and on any one level it was essentially the same whether the other B vitamins were supplied as a synthetic mixture or as a crude rice concentrate. In the presence of m'-methyl-p-dimethylaminoazobenzene the hepatic storage of riboflavin was low on both dietary levels of the vitamin; and previous studies have indicated that tumors due to m'-methyl-p-dimethylaminoazobenzene form at essentially the same rate on either diet.

In the presence of p-dimethylaminoazobenzene more riboflavin was retained in the liver when the fat of the diet was hydrogenated coconut oil than when it was corn oil. Hepatic tumors are known to form more rapidly when the latter oil is fed. In the presence of m'-methyl-p-dimethylaminoazobenzene, however, essentially the same amounts of riboflavin were found in the liver whether corn oil or hydrogenated coconut oil were fed, and on the basal diets used, the nature of the oil does not appear to affect the rate at which liver tumors develop when the m'-methyl dye is the carcinogen. These results, and the quantitative relationship between the carcinogenicity of the many azo dyes and their effects on hepatic riboflavin, suggest that riboflavin retention parallels the ability of the liver to resist the formation of tumors due to azo dyes.

THE LEVELS OF LIPIDS AND CARCINOGENIC AZO-DYES IN THE LIVERS OF RATS FED VARIOUS DIETS CONTAINING p-DIMETHYL-AMINOAZOBENZENE. RELATIONSHIP TO THE FORMATION OF HEPATOMAS. HERBERT SILVERSTONE, and ALBERT TANNENBAUM. (Department of Cancer Research, Michael Reese Hospital, Chicago, Ill.)

The hypothesis that diets affect the formation of azo-dye-induced hepatomas in rats through modifying the level of carcinogenic azo dyes in the liver has been studied. The possibility that carcinogenicity might be influenced by the liver lipid level was also considered. Groups of 24 rats were fed the following diets: (a) brown rice; (b) brown rice plus 15 per cent brewers' yeast; (c) a "synthetic" diet high in protein and fat; (d) a "synthetic" diet low in protein and high in fat; (e) a similar "synthetic" diet low in both fat and protein. Six hundredths per cent p-dimethylaminoazobenzene was incorporated into each of the diets for 4 months; the dye was then omitted and the diets continued until death of the animal or the termination of the experiment 2 months later. The same diets with azo dye were also fed to groups of 5 rats for 7 weeks, following which the animals were sacrificed and their livers analyzed for total lipids, free and total cholesterol, lipid phosphorus, and carcinogenic azo dyes (p-dimethylaminoazobenzene plus p-monomethylaminoazobenzene). The levels of azo dyes appeared to be positively associated with the formation of hepatomas. There was no evidence that either hepatoma formation or the concentration of carcinogenic azo dye in the liver are dependent on the level of liver lipids.

INFLUENCE OF THIOURACIL UPON THE CARCINOGENIC ACTION OF ACETYLAMINOFLUORENE. K. E. PASCHKIS, A CANTAROW, and J. STASNEY. (Jefferson Medical College and Hospital, Philadelphia, Pa.)

Rats fed 2-acetylaminofluorene develop a variety of malignant tumors. We have reported previously that treatment with certain sex hormones hastens and intensifies the development of cancer of the liver by this carcinogen. We have now found that administration of thiouracil protects the liver against the carcinogenic and other effects of acetylaminofluorene and also against the "potentiated" carcinogenicity of combined acetylaminofluorene and testosterone treatment. At the same time there are indications that the androgenic effect of testosterone is more pronounced in animals receiving thiouracil than in those receiving the hormone alone. These findings suggest that the protective action of thiouracil may consist, in part at least, in preventing the transformation of testosterone to a compound of carcinogenic (or co-carcinogenic) and at the same time of lessened androgenic potency.

Thiouracil treatment failed to protect the liver against the carcinogenic effect of dimethylaminoazobenzene.

The thyroid glands of animals treated with acetylaminofluorene and thiouracil show essentially the same changes (hyperplasia, adenoma) as those observed in rats treated over long periods of time with thiouracil alone. Malignancy of the thyroid developed in a few animals treated with acetylaminofluorene and thiouracil. Inasmuch as this has been reported by others in rats treated with thiouracil alone, the carcinogen appears merely to hasten and intensify the development of thyroid malignancy without being essential to it.

THE CARCINOGENICITY OF CERTAIN COMPOUNDS RELATED TO *p*-DIMETHYLAMINO-AZOBENZENE. KANEMATSU SUGIURA. (Memorial Hospital, New York, N. Y.)

Many aminoazobenzene derivatives have been tested for carcinogenic activity in the rat. N,N-dimethyl-*p*-aminoazobenzene and N-methyl-*p*-aminoazobenzene have been found to be equally carcinogenic. They produced cholangiomas and hepatomas in all animals tested in approximately the same period of time. N,N-dimethyl-3'-methyl-*p*-aminoazobenzene was more carcinogenic than the parent compound N,N-dimethyl-*p*-aminoazobenzene; but the N,N-dimethyl-2'-methyl-*p*-aminoazobenzene and N,N-dimethyl-4'-methyl-*p*-aminoazobenzene were very much less active. N,N-diethyl-*p*-aminoazobenzene and all other higher alkyl homologues of N,N-dimethyl-*p*-aminoazobenzene tested failed to produce cirrhosis or neoplastic changes in the liver of the rat when fed in equimolecular amounts.

The investigation has been extended to several compounds of this series which have not been previously tested. Rats were fed a rice diet to which 0.06 per cent of N,N-dimethyl-*p*-aminoazobenzene dissolved in cottonseed oil or molar equivalent amounts of the other compounds were added. The diet was supplemented with a slice of fresh carrot and water daily. Feeding was continued until the animals either succumbed or were sacrificed at the end of the experimental period of 250 days. The results showed the N-methyl-3'-methyl-*p*-aminoazobenzene was at least as carcinogenic as the N-methyl or N,N-dimethyl compound. However, the N-methyl-2'-methyl-*p*-aminoazobenzene and the N-methyl-4'-methyl-*p*-aminoazobenzene were very much less carcinogenic. Although N,N-diethyl-*p*-aminoazobenzene was noncarcinogenic, N-methyl-N-ethyl-*p*-aminoazobenzene was definitely carcinogenic, an indication of the importance of the methyl radical for carcinogenesis. N,N-diethanol-*p*-aminoazobenzene was also noncarcinogenic. The livers of rats fed N,N-dimethyl-4'-hydroxy-*p*-aminoazobenzene had smooth surfaces and histological examination showed no evidence of tumors, bile duct changes, or abnormal regeneration of the ducts and liver cells, or any abnormal nuclear alteration.

American Association for Cancer Research, Inc.

38th Annual Meeting

Hotel Stevens, Chicago, Illinois
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Proceedings of Business Sessions

MINUTES OF THE MEETING OF THE BOARD OF DIRECTORS HELD MAY 15, 1947

The Board of Directors met at 8:30 p.m., May 15, 1947 at the Stevens Hotel in Chicago, Illinois. Drs. Bittner, Brues, Cowdry, Doisy, Furth, Gardner, Huggins, Little, Shear and Taylor were present.

It was voted that the reading of the minutes of the last meeting be waived.

REPORTS OF OFFICERS

The Chairman of the Board exhibited charts showing the number of new members elected and the number of papers presented at the annual meetings of the Association since 1915. By vote of the Board these charts are reproduced here. The Chairman also showed a list of the Officers, Directors and Councillors of the Association since 1915.

The Acting Treasurer announced the receipt during the year of several gifts totalling $51.00, largely in memory of Mrs. Marcella Dill. After brief discussion, it was voted that these and other gifts to the Association be segregated as a special Journal Fund.

The Treasurer's report was read and on the motion of Dr. M. J. Shear, who had been appointed auditor, was accepted.

REPORTS OF COMMITTEES

Program Committee.—Chairman J. J. Bittner reported that all of the papers submitted for presentation were accepted and placed on the program.

Nominating Committee.—Chairman H. C. Taylor, Jr. reported that his Committee had nominated for members of the Board of Directors to serve until 1950: Drs. A. M. Brues, B. L. Coley, E. T. Engle, J. Furth, C. D. Haagensen, J. G. Kidd, C. C. Little and Shields Warren. These names were listed on the proxies sent to the members of the Association by the Acting Secretary. Count of the proxies showed that Drs. Brues, Furth, Little and Warren received the largest number of votes. It was voted, "That the Acting-Secretary cast one vote for the nominees chosen by the members." The new Directors were then declared elected.

Membership Committee.—Chairman Charles Huggins reported that the Association now had 517 active members and 8 emeritus or honorary members.

The nominations for active membership were presented. Fifty-five candidates were recommended for election. They were:

ALBRIGHT, FULLER, M.D., Massachusetts General Hospital, Boston, Mass.

BAUMBERGER, J. PERCY, D.Sc., Stanford University, California.

BECK, LYLE V., PH.D., Hahnemann Medical College, Philadelphia, Pa.

BEGG, ROBERT WILLIAM, M.D., Dalhousie University, Halifax, N. S., Canada.

BENNETT, WARREN A., M.D., Mayo Clinic, Rochester, Minn.

BIERMAN, HOWARD R., M.D., University of California Hospital, San Francisco, Calif.

BOWMAN, RUSSELL O., PH.D., Rhode Island Hospital, Providence, R. I.

BURDETTE, WALTER J., PH.D., M.D., Louisiana State University School of Medicine, New Orleans, La.

CACERES, EDUARDO, M.D., San Marcos University, Lima, Peru.

CURTIS, MAYNIE R., PH.D., Detroit Institute of Cancer Research, Detroit, Mich.

CUTTING, WINDSOR C., M.D., Stanford University School of Medicine, San Francisco, Calif.

DILLER, IRENE COREY, PH.D., The Institute for Cancer Research, Lankenau Hospital, Philadelphia, Pa.

DUNOFF, GREGORY, M.S., 501 Franklin St., Buffalo, N. Y.

DUBNIK, CELIA S., B.A., National Cancer Institute, Bethesda, Md.

DUNNING, WILHELMINA F., PH.D., Wayne University College of Medicine, Detroit, Mich.

ENGEL, R. W., PH.D., Alabama Polytechnic Institute, Auburn, Ala.

FISHBACK, HAMILTON R., Sc.D., M.D., Northwestern University Medical School, Chicago, Ill.

FRIEDMAN, NATHAN B., M.D., Army Institute of Pathology, Washington, D. C.

GARCIA, GERMAN GARCIA, M.D., Hospital Espanol, Mexico, D. F.

GESSLER, ALBERT E., PH.D., Interchemical Corporation, New York, N. Y.

GOLAND, PHILIP P., M.D., University of Pennsylvania, Philadelphia, Pa.

GORDON, MYRON, PH.D., American Museum of Natural History, New York, N.Y.

GREENE, HARRY JONATHAN, M.D., Kings County Hospital, Brooklyn, N. Y.

GYÖRGY, PAUL, M.D., University of Pennsylvania School of Medicine, Philadelphia, Pa.

HAM, ARTHUR WORTH, M.D., University of Toronto, Toronto, Ontario, Canada.

HAUSCHKA, THEODORE SPALTH, PH.D., The Institute for Cancer Research, Lankenau Hospital, Philadelphia, Pa.

HOLLANDER, FRANKLIN, PH.D., The Mount Sinai Hospital, New York, N. Y.

HUMMEL, KATHERINE PATTEE, PH.D., Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

JONES, HOWARD W., JR., M.D., Johns Hopkins University, Baltimore, Md.

733

New Members of the American Association for Cancer Research

by years

FIG. I

Number of Papers Presented at Scientific Sessions of the
American Association for Cancer Research

Year
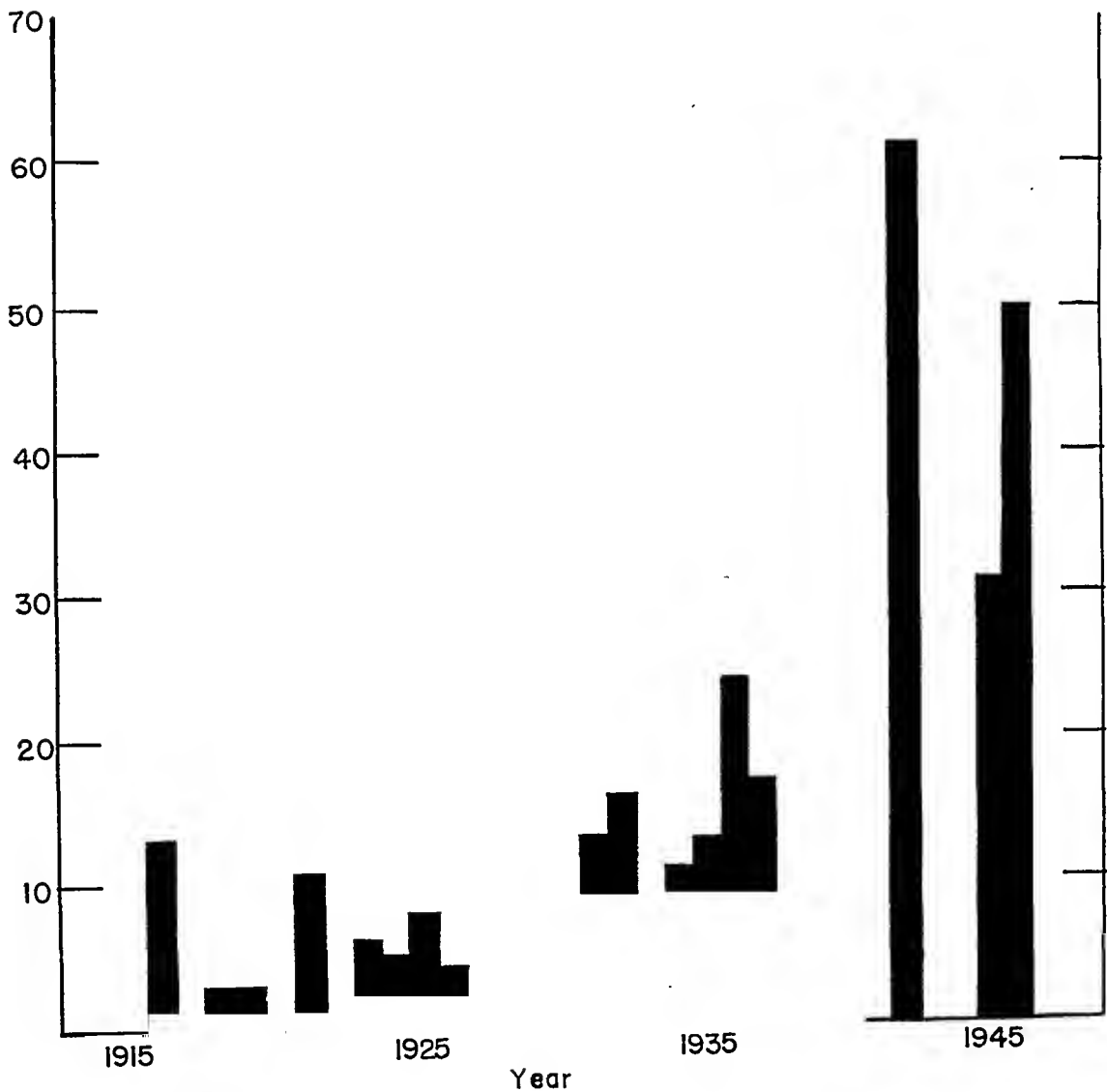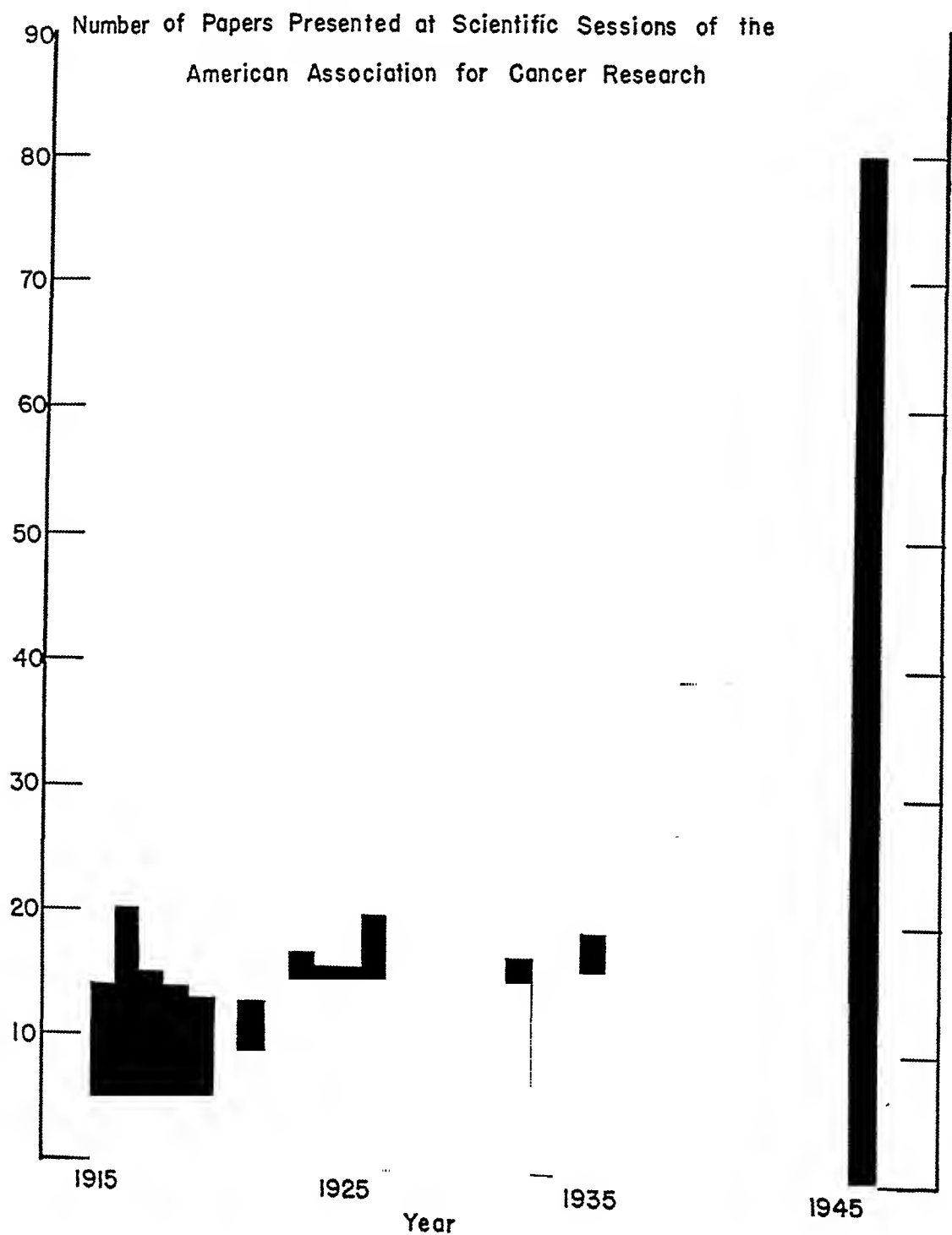
Fig. 2

April 30, 1947

Cash on deposit (Union & New Haven Trust Co.) March 1, 1946		$2441.96
Cash on hand, March 1, 1946		67.00
		2508.96
Checks outstanding, March 1, 1946		20.03
		2488.93
Cash on deposit (Greenwich Savings Bank) March 1, 1946		635.10
Interest on Savings Account to January 1, 1947		9.55
Receipts March 1, 1946 to April 30, 1947		
Dues collected		1607.66
Gifts		51.00
Subscriptions to *Cancer Research*		20.00
		4812.24

Disbursements March 1, 1946 to April 30, 1947			
Secretarial Assistance		$ 150.00	
Printing (Nomination forms, billheads, letterheads and ballots.)		64.00	
Stamps		79.15	
Reprints of By-Laws and list of members	$80.63		
Envelopes and labels for mailing	20.38	101.01	
Meeting March 11 and 12, 1946		287.14	
Registration cards, identification badges and tickets	46.25		
Rental on projector	10.00		
Programs	95.00		
Stenographic service	38.36		
Annual dinner (City tax, gratuities)	88.63		
Expenses of Program Committee	8.90		
Bank charges on foreign checks		1.69	
Subscriptions to *Cancer Research*		20.00	
Assessment for *Cancer Research*			
1946		475.00	
1947		522.00	
Fourth International Cancer Research Congress		512.63	
Contribution	500.00		
Cable and telephone	12.63		
National Society for Medical Research		50.00	
Refund on overpaid dues		6.00	
		$2269.12	$4812.24
Balance April 30, 1947			2543.12
Dues receivable			488.00
			$3031.12

Charles W. Hooker, *Acting Secretary-Treasurer*

I hereby certify that the accounts and vouchers in the American Association for Cancer Research, Inc., for the above recorded period have been examined by me, and that the above are true statements of its financial operations and of its financial conditions as of April 30, 1947.

M. J. Shear, *Auditor for the Directors*

ROBERTS, EUGENE, PH.D., Barnard Free Skin and Cancer Hospital, St. Louis, Mo.

ROBERTSON, WM. V. B., PH.D., College of Medicine, University of Vermont, Burlington, Vt.

RUNNER, MEREDITH N., PH.D., Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

ROYSTER, HENRY P., M.D., University of Pennsylvania School of Medicine, Philadelphia, Pa.

SALOMON, KURT, M.D., PH.D., University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

SCARBOROUGH, J. ELLIOT, M.D., Emory University Hospital, Emory University, Ga.

SELIGMAN, ARNOLD M., M.D., Beth Israel Hospital and Harvard Medical School, Boston, Mass.

SMITH, PAUL KENNETH, PH.D., George Washington University School of Medicine, Washington, D. C.

STASSNEY, JOSEPH, M.D., Jefferson Medical College, Philadelphia, Pa.

STERN, KURT, M.D., Mount Sinai Hospital, Chicago, Ill.

WHITE, PHILIP R., PH.D., The Institute for Cancer Research, Lankenau Hospital, Philadelphia, Pa.

It was recommended that two nominees be elected contributing members:

CARL A. GERSTACKER, B.S.E., Elsa U. Pardee Foundation, Midland, Mich.

ROBERT P. MEADER, M.D., 4002 Jenkins Arcade, Pittsburgh, Pa.

It was recommended that the following distinguished scientists be elected Honorary Members:

DR. E. C. DODDS, London
DR. E. L. KENNAWAY, London
DR. A. LACASSAGNE, Paris

It was voted "That the report of the Committee be accepted and their recommendations be adopted." The nominees were then declared elected as recommended.

Dr. Huggins then presented the recommendation of his Committee with respect to the *qualifications for membership* as specified in the By-Laws. As amended, the recommendation reads:

"The Committee recommends the following changes in the By Laws of the Association:

1. That Section 1 and 2 of Article I of the By-Laws be rescinded.

2. That the following clause be substituted for Section I of Article I:

Section 1, Members.

(a) The Association is to consist of three classes: active members, emeritus members and honorary members.

(b) Candidates for active membership shall be workers in good professional standing who have been active for at least two years in cancer research or for three years in research with one year in cancer research. Any person with these qualifications who has conducted and published meritorious researches in the field of cancer research and who is a resident of the Americas shall be eligible for active membership in the Association.

(c) Emeritus members are those who have attained the age of 65 years and have been members for ten years or more. They shall be exempt from dues.

(d) Distinguished scientists and others who have contributed to the advance of cancer research shall be eligible for election as honorary members of the Association. Honorary members shall be exempt from dues; they shall have the right to attend the meetings of the Association and of taking part in its scientific discussions, but they shall have no vote.

3. That Section 2 of Article I be amended to read:

Section 2. Election of Members.—The Board of Directors at any time and from time to time may elect to membership persons who meet the qualifications listed in Section 1.

4. That the changes in the rules regarding membership be made not retroactive from May 1947."

In the discussion of the proposal it was pointed out that under Section 1, (c) the transfer to Emeritus Membership becomes automatic and does not necessitate application by the member as heretofore.

It was voted "That the recommended changes in the By-Laws be approved and adopted." It was also voted "That the Membership Committee be given a hearty vote of thanks for a splendid job."

The Acting Secretary reported that applications for transfer to Emeritus Membership had been received from Dr. W. F. Jacobs and Dr. E. B. Krumbhaar. The transfers were approved.

The resignations of four members were accepted:

ALVIN J. COX, San Francisco, California
CARL F. SIEKMAN, Buffalo, New York
LESTER F. WICKS, St. Louis, Missouri
MELVIN C. REINHARD, Buffalo, New York

The deaths of the following members were regretfully announced.

HALSEY J. BAGG
STARLING W. CHILDS
ARTHUR E. HERTZLER
BARNET JOSEPH
WARD J. MACNEAL
GEORGE H. SEMKEN
BURTON T. SIMPSON
ROLLIN H. STEVENS

Journal Committee.—Chairman M. J. Shear reported that his Committee met in Philadelphia on February 13, 1947. The approximate cost of publishing *Cancer Research* in 1946 was $16,500. For the same year there were 895 subscriptions. Approximately 230 subscriptions were at $5.00 (yielding $1,150) and 660 at $7.00 (yielding $4,620); the total received from subscriptions was thus $5,770. The Association contributes to the journal $1.00 of the $3.00 dues paid by the 530 members. The total income was, then, about $6,300, leaving an apparent deficit of about $10,000.

The deficit has been met by contributions from the Donner Foundation, The Jane Coffin Childs Fund, and the Anna Fuller Fund. The Pardee Fund has asked to participate, and W. W. Allen of that Fund has been appointed to the Advisory Board of *Cancer Research*.

The Journal Committee has raised the following questions:

(1) Would it be advisable to raise the dues from $3 to $7, giving the journal $5 instead of $1, and giving each member, automatically, a paid subscription to *Cancer Research*? At present, members who subscribe are paying $8 instead of the proposed $7.

(2) Since only 182 of the 530 members subscribe, should an effort be made to stimulate subscriptions by members if the above question receives an adverse response?

(3) Should an effort be made to conduct a subscription drive among scientists, clinicians and others who might subscribe if the journal were called to their attention in an effective fashion?

The Committee viewed with approval the idea of attempting to establish an endowment for the journal. It also recommended that the Association encourage its members to submit more of their original papers to *Cancer Research*, and that it authorize the Editor to stimulate competent workers to prepare reviews on selected subjects for the journal.

Now that the subscription list is approaching 1,000, the Business Manager has been requested to investigate the possibility of securing advertisements.

The question given greatest attention was that of increasing the annual dues of the members and making subscriptions automatic. It was voted "That the proposal be referred to the members of the Association." It was also voted, with one Director dissenting, "That a favorable recommendation of the Board of Directors accompany the referral."

UNFINISHED BUSINESS

The Chairman reported the results of his inquiry into the possibility and desirability of the Association's affiliating with the Federation of American Societies for Experimental Biology. It was voted "That consideration of affiliation with the Federation be terminated."

It was disclosed that no financial report of the last International Cancer Research Congress is available.

The Chairman reviewed the desirability of having a history of the Association prepared and reported that Dr. W. H. Woglom declined appointment as historian. It was voted "That the Chairman appoint a Committee to obtain from the older members of the Association data and their recollections on the early history of the Association." This action was regarded necessary in view of the lack of data from 1907, the year of organization, to 1915. Dr. C. C. Little was appointed Chairman of the committee and instructed to select his own associates.

NEW BUSINESS

The World Health Organization and the desirability of the Association's participation were discussed briefly. It was decided that the next Chairman of the Board appoint a committee to formulate recommendations.

Nominations for officers for the coming year were then made; For President, John J. Bittner; for Vice-President, Charles Huggins. It was voted "That Charles W. Hooker be appointed Secretary-Treasurer for the coming year and be made ex-officio a member of the Board of Directors."

It was voted that the costs of conducting the annual meeting be paid from the funds of the Association.

The meeting was adjourned at 12:00 midnight.

WILLIAM U. GARDNER,
Chairman, Board of Directors
CHARLES W. HOOKER,
Acting Secretary

MINUTES OF THE MEETING OF THE MEMBERS HELD MAY 16, 1947

The meeting of the members of the Association was called to order at 1:45 p.m., May 16, 1947 at the Stevens Hotel in Chicago, Illinois.

Reading of the minutes of the last meeting was omitted on vote of the members.

The reports of the Acting Treasurer and Auditor were presented and approved.

The results of the count of proxy votes for new Directors to serve until 1950 and the action of the Board of Directors were reported as recorded in the minutes of the meeting of the Board.

The nominations for officers of the Association made by the Board of Directors were read:

JOHN J. BITTNER, *President*
CHARLES HUGGINS, *Vice President*
CHARLES W. HOOKER, *Secretary-Treasurer*

The candidates were considered separately and elected.

The list of newly elected members was read by the Secretary. These names are recorded in the minutes of the meeting of the Board of Directors on May 15, 1947.

The change in the By-Laws concerning qualifications for membership adopted by the Board of Directors was read. It was voted "That the change be approved."

The proposal that the annual dues of members be increased from $3.00 to $7.00 and include a subscription to *Cancer Research* was presented and discussed. It was voted "That the desire of the members be ascertained by distribution of printed ballots by mail."

The meeting adjourned at 2:31 p.m.

WILLIAM U. GARDNER,
President
CHARLES W. HOOKER,
Secretary

MINUTES OF THE MEETING OF THE BOARD OF DIRECTORS HELD MAY 17, 1947

The meeting was called to order at 12:42 p.m. at the Stevens Hotel in Chicago, Illinois, following waiver of previous formal notice of the meeting of the Directors signed by all Directors present and constituting a quorum. Present were Directors Aub, Bittner, Brues, Cowdry, Doisy, Gardner, Huggins and Shear.

The question of publication of abstracts prior to the annual meeting was again discussed. It was voted "That the present procedure be continued next year and that the Program Committee submit recommendations as to the make-up and handling of the program." The question of symposia being held in conjunction with the annual meeting was also discussed and it was voted "That consideration of symposia be made the responsibility of the Program Committee for decision and action."

Dr. W. U. Gardner was appointed chairman of a committee to investigate the World Health Organization.

Dr. C. C. Little was appointed chairman of a committee on memorials for deceased members.

It was voted "That Chairman appoint a member of the Association to examine the desirability of affiliating with the Union of Biological Societies and submit a report."

NEW BUSINESS

It was unanimously resolved, "That Charles W. Hooker, Secretary-Treasurer and John J. Bittner, President, be, and each of them hereby is authorized in the name and on behalf of the Corporation to open a bank account or bank accounts with such banks, bankers and/or trust companies as they or each of them shall determine, and to deposit therein to the credit of the Corporation from time to time any and all monies and checks of the Corporation; and

Resolved, that the banks, bankers, and/or trust companies so designated as depositaries of the Corporation be, and they hereby are, severally authorized to honor and pay all checks, drafts, and other orders for the payment of money drawn upon such account or accounts (including checks, drafts, or other orders of one or both of the persons making, signing, or drawing them) made, signed or drawn by the following persons: Charles W. Hooker or John J. Bittner."

It was also *"Resolved*, that the Greenwich Savings Bank of 1356 Broadway and 985 Sixth Avenue, Borough of Manhattan, City of New York, is hereby designated as depositary of funds of this corporation and is authorized to honor drafts and orders for the payment and withdrawal of moneys therefrom made in the name of this corporation and signed by President, John J. Bittner, or Secretary-Treasurer Charles W. Hooker.

"And it is Further *Resolved* that the foregoing authority shall continue until written notice of revocation of this Resolution shall be received by the Greenwich Savings Bank.

"And it is Further *Resolved* that said The Greenwich Savings Bank is authorized to accept the certificate of the Secretary of this corporation as evidence of the names and signatures of the persons at any time authorized to act pursuant to this Resolution."

It was voted "That the sum $250 be allocated for secretarial assistance to be used as necessary."

It was voted "That the publication of the minutes and the scientific proceedings of the meeting be authorized and that the cost of publication be paid by the Association."

The Chairman of the Board proposed the following standing committees:

Program Committee.—J. C. AUB, *Chairman:* A. M. BRUES, E. A. DOISY.

Nominating Committee.—W. U. GARDNER, *Chairman:* W. E. HESTON, ALBERT TANNENBAUM.

Membership Committee.—CHARLES HUGGINS, *Chairman:* A. M. BRUES, J. FURTH.

Journal Committee.—M. J. SHEAR, *Chairman:* S. BAYNE-JONES, M. W. S. SCHRAM.

Cancer Research, its Organization and Support.—SHIELDS WARREN, *Chairman:* CHARLES HUGGINS, G. M. SMITH.

The Board approved the proposed Committees.

It was voted "That the report of the *Committee on Cancer Research, its Organization and Support* be published as part of the minutes of the meeting." The report, which was read at the annual dinner, follows:

"The Committee on Cancer Research, its Organization and Support, felt that its first responsibility was to determine the attitude of those carrying on cancer research, the members of this Association, toward the Association taking a more active part in such matters and to determine their views with regard to forms of support for cancer research recently or currently planned.

"The questionnaire sent to the members is reproduced herewith and the replies tabulated. Many members sent additional comments, which have been used to help formulate additional recommendations.

"To summarize the viewpoint of the membership as a whole as presented by the questionnaire, there is a predominant feeling that the Association should actively interest itself in recommendations for the financial support of cancer research and also a federal subsidy is desirable. However, a lump sum appropriation is opposed. It was felt that no federal funds for cancer research should be administered through state health departments. The majority of members felt the present method of disbursement of federal funds for cancer research and of funds for cancer research by the American Cancer Society through the Committee on Growth to be satisfactory. However, approximately one-third of those voting felt that the present methods were not entirely satisfactory.

"It is obvious that most members desire their Association to take an active part in the organization and support of cancer research.

"The formal organization of cancer research, or of any research, is of uncertain value. Only when fundamental principles are known, and objectives clearly defined in relation to this knowledge can organization be expected to yield fruitful results. Organization of research to prevent duplication, to guide development, tends to sterility and to the production of "pot-boilers." Such organization might well have prevented the discovery of insulin on the ground that the experiments had been done before.

"Cancer research has not yet reached and probably will never reach the stage where organization of a directional type can be other than hampering. Organization for the dissemination of knowledge in the field is desirable. Increased support should be given our official journal, *Cancer Research*. Conferences such as those sponsored by the American Cancer Society, the Donner Foundation and others, of those working in specialized fields are of value, as well as such general conferences as our projected Fourth International Cancer Research Congress next September.

"Support of Cancer Research should be continuing rather than on a lump sum basis with a short time limit, as in the proposed Pepper-Nealy Bill of the last session.

The problem of cancer is not so nearly solved that a time limit can be set for final success. The system of annual grants has been widely recognized as unsatisfactory, and most major grants-in-aid are now made with a tacit if not actual understanding that support will continue for several years.

"Two distinct types of research aimed toward the solution of the cancer problem exist—work on fundamental biological, chemical and physical problems that may shed light on abnormal growth and its control, and work directly aimed at understanding and controlling neoplastic growth. At the present the former type is receiving greater emphasis in part because many investigators believe cancer can be understood only through broad advances in biology, in part because of lack of data to permit a frontal attack, in part because some of those bringing new knowledge and new techniques to bear on the cancer problem have little or no knowledge of the disease.

"One of the most acute problems at present is to conserve for continued cancer research the young and able investigator as he matures. The present system of grants-in-aid provides adequately for investigators of fellowship grade, but when a worker of proved ability reaches his late thirties or forty, annual grants and salaries less than those of bus drivers or brick-layers are insufficient. A heavy loss of highly trained research workers is thus inevitable. Special effort should be made to provide means of encouraging these to remain in the cancer research field and to capitalize on their experience."

It was voted "That the next annual meeting be held in conjunction with the meeting of the Federation of American Societies for Experimental Biology, preceding that meeting if possible."

With respect to the program it was agreed that there be no change in the current practice of designating non-members as presenting papers "By invitation". It was also agreed that the Program Committee should make recommendations as to the style of abstracts acceptable.

Concern was expressed over the interruption of publication of *Cancer Research*. After discussion,

It was Resolved "That the Board of Directors of the American Association for Cancer Research appreciates the difficulties facing the journal, but wishes to emphasize the urgency of prompt resumption of publication in order that the accelerated research in cancer be made available to investigators."

The meeting was adjourned at 1:30 p.m.

JOHN J. BITTNER,
Chairman, Board of Directors

CHARLES W. HOOKER,
Secretary

RESULTS OF THE VOTING

	Yes	No	No vote
1. (a) Should the Association limit its activities to the publication and interchange of scientific information?	42	119	97
or			
(b) Should the Association also be interested in recommendations for the financial support of cancer research?	223	23	12
2. Do you favor a Federal subsidy for cancer research?	225	27	6
3. Is a Federal lump sum appropriation of $100,000,000 for cancer research the most satisfactory type of support?	101	107	50
4. Should the Association make recommendations for the administration of such an appropriation?	223	27	8
5. Should Federal funds for cancer research be made directly available to			
(a) Institutions for cancer research	219	18	21
(b) Universities	214	22	22
(c) Hospitals	183	40	35
(d) Individual investigators	185	49	24
6. Should Federal funds for cancer research be administered by state health departments?	21	231	6
7. Should the Association seek representation in any Federal agency established to distribute cancer research funds?	219	32	7
8. Is it your opinion that the Association should have representation in other national public agencies set up to collect funds from the public to distribute them for cancer research?	213	42	3

	Satisfactory	Unsatisfactory	No Opinion
9. Do you consider the method of disbursement of Federal funds for cancer research on recommendation of the National Advisory Cancer Council of the United States Public Health Service as satisfactory, unsatisfactory, or have you no opinion?	114	50	94
10. Do you consider the method of disbursement of funds for cancer research by the American Cancer Society through the Growth Committee of the National Research Council as satisfactory, unsatisfactory, or have you no opinion?	108	53	97

The Transplantability of Mammary Cancer in Mice Associated with the Source of the Mammary Tumor Milk Agent*

John J. Bittner, Ph. D.

(From the Division of Cancer Biology, Department of Physiology, University of Minnesota Medical School, Minneapolis 14, Minnesota)

The experiments on the transplantation of tumors by Little and Tyzzer (31), Strong (36), Strong and Little (40) and Little and Strong (30) resulted in the advancement of the genetic theory of transplantation. According to this hypothesis, susceptibility or non-susceptibility to grafts of tumor tissue is dependent upon the genetic relationship between the host inoculated and the tumor cell. The growth of leukemia (32, 34, 20), other types of tumors (see 27 and 28), and the retention of normal tissue, such as spleen (29, 12), may be explained upon the same basis.

Sex-linkage (39) and linkage with color genes has been indicated (30) and demonstrated (5-9). Several investigators (37, 38, 4, 18, 19) have detected mutations in the genetic constitution of the transplantable mammary tumors. Whereas multiple mammary tumors from a single animal have never required the same genetic make-up for progressive growth (39, 4, 18, 19, 5-9), animals inoculated with multiple grafts of the same tumor responded by either growing all or none (3). Gorer (21-23) has evidence that there may be a relationship between some of the genes necessary for the growth of some transplanted tumors and those which determine the presence of an antigen. The protective antibodies he considers to be iso-antibodies.

Spontaneous mammary cancer in mice usually results from the action of three primary factors (14). One of these is the mammary tumor milk agent (11) which because of its small size, ability to propagate in the living cell, and its antigenic properties may be classified as a filterable agent or virus (see 14-16, 35, 2, for literature).

In various reciprocal crosses between high cancerous strains of mice different incidences have been noted in the hybrids depending upon the maternal stock. In some crosses these differences were noted when the mice were continued as breeders (10), in others only the virgin hybrids showed the variation (33, 16, 41, 17). How these data may be interpreted at this time is problematical. If the agents from two high cancerous strains might be considered to be the same, then they have different activities when obtained by hybrid mice with the same genetic constitution; if the agents are not the same, then the hybrids with identical susceptibilities for spontaneous mammary cancer, perhaps expressed in part through hormonal stimulation, produce different activity as determined by the incidence of mammary cancer and average cancer age. These in turn may be altered by changing the degree of hormonal stimulation (16, 17).

In this study we have investigated the transplantability of spontaneous mammary cancer primarily from the standpoint of the source of the milk agent.

MATERIALS AND METHODS

In Table I we have listed the mammary tumors which were transplanted, the strains in which they developed and the strains from which the animals obtained the milk agent. The tumors were inoculated subcutaneously by the trochar method.

The various strains of mice tested with implants of these tumors are too well known to require description and are given in the various tables. However, some lines of these strains had been nursed by females of other stocks so that they had obtained either the mammary tumor milk agent from the fostering mothers or the milk agent had been eliminated by the same process.

TABLE I: Gives the Origin of the Mammary Tumors Which Were Transplanted and the Source of the Milk Agent

Tumor inoculated	Stock of origin	Source of milk agent
Az #7666	A stock	Z (C3H) stock
Za #7667	Z (C3H) stock	A stock
Za #7727	Z (C3H) stock	A stock
AaZF₁ #7668	A♀ × Z♂ F₁	A stock
ZzAF₁ #7665	Z♀ × A♂ F₁	Z stock
D₂ #5687	D stock—line 2	D₂ line
D₁ #5736	D stock—line 1	D₁ line
D₂ #6086	D stock—line 2	D₂ line
D₈ #6087	D stock—line 8	D₈ line
D₁ #6088	D stock—line 1	D₁ line
B₆ #7106	B stock—line 6	A stock
B₆ #7476	B stock—line 6	A stock
B₆ #7477	B stock—line 6	A stock

RESULTS

The results obtained following the inoculation of spontaneous mammary carcinoma from the A and Z (C3H) cancerous strains and their reciprocal hybrids are tabulated in Table II.

the Z stock, descended from 4 mothers of which 2 were litter mates, proved to be resistant but were found to be susceptible to another Za tumor, No. 7727. Ten Aa and 3 Ax mice, negative to tumor Za No. 7667, were reinoculated with the Az tumor, No. 7666 and all grew that tumor progressively.

The tumors from the reciprocal hybrids between the A and Z strains, AaZF₁ No. 7668 with the milk agent from the A stock and ZzAF₁ No. 7665 with the Z milk agent, failed to grow in any of the mice of the two parental stocks regardless of milk agent. They both grew progressively in all hybrids of the A × Z cross, in mice with or without the agent. A few hybrids that had one parent from either the A or the Z strain were inoculated and they were all resistant to the two hybrid tumors (Table II).

Several mammary tumors were transplanted which arose spontaneously in breeding females of the dilute brown stock. Several sublines of this stock

TABLE II: Results Obtained Following the Transplantation of Mammary Cancer From the A and Z (C3H) Stocks and Their Reciprocal Hybrids (A Tumor with Z Agent, No. 7666: Z Tumor with A Agent, No. 7667; and Reciprocal Hybrid Tumors with Either A, No. 7668; or Z Milk Agent, No. 7665)

Stock inoculated	Genetics of hosts	Milk agent	Az +	7666 −	Za +	7667 −	AaZF₁ +	7668 −	ZzAF₁ +	7665 −
Aa	A stock	A stock	27	0	0	15*	0	11	0	10
Az	A stock	Z stock	4	0	0	5	0	4	0	6
Ax	A stock	None	9	0	0	19*	0	0	0	17
Zz	Z stock	Z stock	0	6	12	0	0	0	0	6
Za	Z stock	A stock	0	3	6	5*	0	0	0	8
Zb	Z stock	None	0	17	19	4*	0	4	0	10
AaZF₁	A♀ × Z♂	A stock	3	0	3	0	23	0	6	0
ZzAF₁	Z♀ × A♂	Z stock	0	0	22	0	4	0	8	0
ZaAF₁	Z♀ × A♂	A stock	11	0	5	0	0	0	11	0
AxZbF₁	Ax♀ × Zb♂	None	33	0	6	0	15	0	5	0
ZbAxF₁	Zb♀ × Ax♂	None	5	0	4	0	32	0	23	0
AaDF₁	A♀ × D♂	A stock	9	0	0	9	0	6	0	6
AaIF₁	A♀ × I♂	A stock	4	0	0	4	0	8	0	4
ZzDF₁	Z♀ × D♂	Z stock	0	4	4	0	0	0	0	0
DdZF₁	D♀ × Z♂	D stock	1	5	5	0	0	4	0	0
DdAF₁	D♀ × A♂	D stock	7	0	0	5	0	0	0	2
AxCeF₁	Ax♀ × Ce♂	None	10	0	0	5	0	0	0	5
ZbDF₁	Zb♀ × D♂	None	0	0	8	0	0	8	0	0

* See text.

Tumor Az No. 7666 developed in a female of the A strain with the milk agent from the Z stock and when transplanted grew progressively, resulting in the death of the hosts, in all the animals of the A strain and in F₁ hybrids which had one parent from the A strain. Whether or not these animals had the milk agent made little difference. One exceptional inoculation was noticed in that one of the six F₁ hybrids between Z and D (dilute brown) strains was susceptible to the tumor from the A stock.

Only animals of the Z (C3H) strain or hybrids derived by mating Z mice to animals of other stocks responded by being susceptible to the tumor, No. 7667, which developed spontaneously in a female of that strain with the A milk agent. Nine mice of

were tested and the source of the lines were: sublines 1 or 12 and 2 or 212, G. W. Woolley, Bar Harbor, while the mice referred to as line 8 had been secured from S. G. Warner, Springville, N. Y. The results are given in Table III.

The mice of line 2 of the dilute brown stock and their F₁ hybrids (D₂ × Z stocks) showed progressive growth of grafts of the 2 tumors that arose in mice of that line but they were all resistant to 2 tumors from line 1 and 1 tumor from line 8. The 2 tumors from line 1 produced transplanted tumor in all but one mouse of that subline and their F₁ hybrids. Six of the 10 mice from line 1 were susceptible to a tumor from a mouse of line 8 but none grew the tumors from line 2. Mice of line 8 were

susceptible to grafts of the tumor from line 8, resistant to the tumor from line 2, and 1 of 16 grew the tumor from line 1.

Four sublines of the C57 black or B stock were tested to implants of mammary tumors from that stock. They were, with the source of the original materials: line 4, W. L. Russell, Bar Harbor; line 6, E. Fekete, Bar Harbor; and lines B and D, S. G. Warner, Springville. The mammary tumors arose in mice of line 6 of the B stock which had the milk agent from the A stock. Tumor No. 7106 developed

time these studies were completed, it has become quite evident that the growth of these mammary tumors was not associated with the presence of the milk agent. Also, had the action of the milk agent been primarily responsible for the growth of such tumors, multiple primary tumors from a single host would have been expected to have given identical results when transplanted. That is, the inoculation of animals of the generations where segregation of the determining genes takes place would be expected to grow either all or none of these primary

TABLE III: RESULTS OBTAINED FOLLOWING THE INOCULATION OF SEVERAL MAMMARY TUMORS FROM MICE OF SUBLINES OF THE D OR DILUTE BROWN STOCK INTO ANIMALS OF THESE LINES AND F₁ HYBRIDS

Stock Inoculated	Genetics of hosts	Milk agent	Tumor #5687 D—Line 2		Tumor #5736 D—Line 1	
			+	−	+	−
D — Line 2	D_2	D_2 stock	35	0	0	12
D — Line 1	D_1	D_1 stock	0	38	6	0
ZbD₁ F₁	Zb × D_1	None	0	15	15	0
ZbD₂ F₁	Zb × D_2	None	11	0	0	11
ZzD₁ F₁	Zz × D_1	Z stock	0	8	8	0
ZzD₂ F₁	Zz × D_2	Z stock	6	0	0	6

			Tumor #6086 D—Line 2		Tumor #6087 D—Line 8		Tumor #6088 D—Line 1	
			+	−	+	−	+	−
D — Line 2	D_2	D_2 stock	33	0	0	33	0	30
D — Line 8	D_8	D_8 stock	0	25	25	0	1	15
D — Line 1	D_1	D_1 stock	0	10	6	4	9	1

in a mouse of the fostered generation whereas tumors No. 7476 and No. 7477 occurred in the progeny of the fostered animals. These tumors were inoculated into mice of the B stock which had not been fostered and thus would not be expected to have the mammary tumor milk agent. No negative animals were observed, regardless of sublines, to the grafts of the three C57 black tumors (Table IV).

TABLE IV: TRANSPLANTATION OF MAMMARY TUMORS FROM MICE OF THE B (C57 BLACK) STOCK WITH THE A MILK AGENT INTO B MICE WITHOUT THE AGENT

Stock inoculated	Tumor #7106 +	−	Tumor #7476 +	−	Tumor #7477 +	−
B — Line 4	26	0	0	0	0	0
B — Line 6	5	0	45	0	41	0
B — Line B	5	0	0	0	0	0
B — Line D	12	0	11	0	11	0

DISCUSSION

The genetic theory of transplantation was advanced only after inbred strains of mice had been developed. By these studies it has been possible to detect genetic changes in the tumor cell (37, 38, 4, 18, 19) and to determine the homozygosity of strains or sublines of the same strain (25, 26).

Mammary tumors that developed in F₁ hybrids derived by crossing 2 high cancerous strains of mice were found to grow in all of the F₁ hybrids but in few, if any, of the parental stocks (4-9). Although the milk agent had not been demonstrated at the

tumors when they were grafted simultaneously. These results have never been realized in that two primary tumors from a single host have never been found which required identical genes for progressive growth (39, 4, 18, 19, 4-9).

In this report we have considered the transplantation of spontaneous mammary carcinoma which arose in mice of 2 inbred strains of mice after the animals had obtained the milk agent from the other stock by foster nursing. Previous studies (16, 17) showed that the milk agent from the two donor strains did not have the same activity (tumor incidence and average cancer age) in mice with the same genetic susceptibility for spontaneous mammary cancer. If the growth of these tumors, when transplanted, had been dependent entirely upon the presence and "type" of milk agent, the following results might have been obtained:

1. If the milk agent from the 2 stocks were the same, all animals with the agent would be expected to be susceptible while mice of the same genetic constitution but without the agent should be resistant.

2. If the milk agents from the A and Z strains were not identical, the tumor from the A stock with the Z agent would be expected to grow in all mice with the Z agent and the tumor from the Z stock with the A agent should give progressive growth in mice with the A agent.

3. The growth of tumors from the reciprocal hybrids would be dependent upon the presence of the milk agent and respond accordingly.

The results showed, however, confirming the observations of others, that the transplantability of these mammary tumors was not dependent upon the presence of the milk agent. The tumors grew equally well in animals that lacked the agent as in those that had it and in mice possessing an agent from a different source (*i.e.*, a different strain) as in animals with the agent from the same source as was present in the tumor. The reaction of the host was dependent upon the genetic relationship between the host and the tumor cell.

The application of these findings to the growth of spontaneous mammary cancer in mice would be entirely theoretical. If cancer develops as the result of a somatic mutation, as has been suggested by many investigators, any cell capable of becoming cancerous but with a genetic constitution incompatible with that of the host would not be expected to survive. For obvious reasons it would be impossible to obtain such data.

Although the mice of all of the sublines of the C57 black stock responded alike to grafts of the 3 tumors from that stock, this does not imply that they had the same susceptibility for spontaneous mammary cancer. Others have found that the incidence of spontaneous mammary cancer may range from approximately 10 per cent (1, 15) to 76 per cent (24) in mice of the various sublines of this strain when they have the milk agent. Mice of at least 2 of the lines used will develop mammary cancer if they possess the agent.

In the dilute brown stock different incidences of spontaneous mammary cancer are being obtained in mice of the various sublines. Thus, they may have different susceptibilities for the growth of transplanted mammary cancer as well as for spontaneous mammary cancer. It has never been suggested that these may be comparable.

SUMMARY

The growth of mammary cancer in mice, as determined by its transplantability, is dependent upon the genetic relationship of the host and the tumor cell and not the mammary tumor milk agent.

The theoretical application of these results to the growth of spontaneous mammary cancer was considered.

Genetic differences in sublines of the same stock to transplanted mammary tumors may or may not indicate genetic variations in the inherited susceptibility for spontaneous mammary cancer.

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Thyroidal and Vascular Changes in Mice Following Chronic Treatment with Goitrogens and Carcinogens

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In recent years understanding of some of the factors involved in atypical growth of carcinogenesis of several endocrine organs has been advanced significantly. The thyroid gland, despite its comparatively simple embryonic and adult morphology, and despite its presumably simpler interrelationships with other endocrines has not benefited by these advances.

Concerning tumors of the human thyroid a voluminous literature, mostly descriptive, has arisen. Wegelin (44) first made a distinction, on the basis of origin, between those tumors derived from the lateral (ultimobranchial) embryonic anlage of the thyroid, and those derived from the median component. This distinction has been amplified and supported by many pathologists (7, 8, 11, 20, 26, 42), although discounted by some (19). An interesting and probably significant observation is the fact that almost all human thyroid malignancies originate in hypoplastic glands, usually from small, benign, adenomatous nodules (13, 25, 27, 44). Pemberton reports (27) that only 10 of the 774 cases of thyroid carcinoma noted by him could be said to have arisen in hyperplastic thyroids. Another common characteristic of human thyroid cancers is the relatively high frequency of their metastatic establishment in the lungs and in the venous channels and heart en route to the lungs (7, 15, 32, 42, 43). Thorek (37) assigns this property of vascular migration to a pulmonary site, also to some thyroidal growths which he classifies as benign adenomas. The case which he describes has interesting counterparts in some of the experimental mice described in this report.

Spontaneous thyroidal tumors in non-primate and non-mammalian vertebrates are characterized by their rareness (5, 35). Some of those reported, especially the so-called carcinomas of fish thyroid, are probably benign adenomas (12, 24).

In mice Slye (34) found only 17 spontaneous malignant thyroidal growths in 61,700 animals examined. Others have described single specimens of rats or mice with carcinoma of the thyroid (6, 23, 35).

Slye (34) has provided data indicating that the rare tumors of the thyroid in mice have a genetic basis. Experimental work dealing with atypical growth in the thyroid has been of several categories. Some investigators have been concerned with the effect on the thyroid of spontaneous or implanted tumors in another part of the body, or of distantly applied chemical carcinogens (2, 21, 41). Responses to such treatment vary from none, to mild epithelial and secretory stimulation. Barry and Kennaway (2) found this type of response to be quite variable among different strains of mice.

Esmarch (10) by depositing methylcholanthrene in contact with the thyroids of rats was able to induce formation of 3 sarcomas and 3 squamous epitheliomas in the region of treatment.

The use of goitrogenic drugs and diets has provided another means for study of atypical thyroidal growth. Hellwig (17) produced thyroid adenomas in rats given iodine-poor, calcium-rich diets. Bielschowsky (3, 4) by feeding the goitrogen thiourea with carcinogen 2-acetyl-aminofluorene, produced nodular adenomatous thyroids in rats. Griesbach, Kennedy, and Purves (14) obtained similar growths by feeding Brassica seed diets alone for as long as 41 weeks, and Purves and Griesbach (28, 29) by feeding thiourea alone to rats for almost two years observed thyroidal growths which they consider malignant.

Van Dyke (38) found a high normal incidence of thyroidal cystadenomas in older rats, accompanying involutional changes. He presented evidence indicating that these cystadenomas are of ultimobranchial origin and that their incidence could be increased in younger animals when thyroid involution was induced by feeding a choline-deficient diet (39).

The experiments to be reported here were begun before the appearance of the work of Bielschowsky and Griesbach (3, 14). They deal with mice of several inbred genetic strains kept for as long as 82 weeks on goitrogenic diets containing thiourea or thiouracil. To some of the mice on such diets,

the carcinogen benzpyrene was administered subcutaneously, and to some, after the appearance of Bielschowsky's publication (3), 2-acetyl-aminofluorene was fed. It is hoped that this report will add to the understanding of the relation of goitrogens to possible carcinogenesis. It differs from the previous publications in (a) dealing with mice instead of rats, (b) the length of treatment, (c) the description of the histological changes predisposing to blood vessel "invasion" by thyroid, (d) the investigation of the effect of discontinuing the goitrogenic diet after advanced stages of atypical growth have been achieved, and (e) the attention paid to the behavior of ultimobranchial tissue during these changes. Also, it is possible to speak of differences mediated by genetic factors since several inbred strains were tested.

thiourea were given a tracer dose of 15 μc. of radioactive I¹³¹ 24 hours before autopsy.

All thyroid glands were fixed in Bouin's fluid and serially sectioned. Sections were stained with hematoxylin and eosin. Lungs, when taken, were given the same treatment. Pituitaries were fixed in 10 per cent formalin and stained with a modified Mallory stain (33).

Unstained serial sections of thyroids from animals given radio-iodine were exposed to x-ray film for preparation of radio-autographs according to the method described by Hamilton, Soley, and Eichorn (16). The sections were afterward stained with hematoxylin and eosin.

Two groups of 6 animals from both the thiourea and the thiouracil feeding experiments were returned to the stock (Purina fox chow) diet for a

TABLE I: NUMBERS OF ANIMALS EXAMINED IN EACH PHASE OF EXPERIMENTAL TREATMENT

Genetic strain	A	C₅₇	I	A×C₃H† hybrid	A×CBA† hybrid
Untreated control	36	51	35	4	4
2% Thiourea	31 (53)*	43 (65)*	17 (24)	9	4
0.1% Thiouracil	23 (28)	26 (29)	20 (24)		
2% Thiourea, 1 mgm. benzpyrene	26 (41)	20 (24)			
1.0% Thiourea, 0.05% aceto-aminofluorene	19 (24)	17 (18)	19 (24)		
2% Thiourea, estradiol				9	4
Estradiol					7

* Figures in parentheses indicate numbers of animals at beginning of experiment.
† Thyroids of mice in these groups obtained from animals treated for another purpose by Dr. W. U. Gardner.

MATERIALS AND METHODS

A total of 434 mice, including 122 untreated controls, was examined, of an original 527 animals started in the experiment. The apportioning of animals of different strains to various phases of the investigation is detailed in Table I. Mice were all between 1 and 3 months of age at the beginning of treatment.

The 2 per cent thiourea diet was prepared by adding finely ground thiourea (Eastman Kodak) to powdered Purina fox chow. The 0.1 per cent thiouracil diet was prepared for feeding in a similar fashion, as was the 1.0 per cent thiourea-0.05 per cent acetoaminofluorene diet.[1] Forty-six of the animals included in this report were given 1.0 mgm. of benzpyrene in olive oil in a single subcutaneous injection about 30 days after first being placed on the 2 per cent thiourea diet. Estradiol was administered either by subcutaneous injection in sesame oil, or by subcutaneous implantation of a pellet.

Twelve mice selected from the group receiving

week preceding autopsy to learn whether the induced thyroidal changes were reversible.

Tumors, which frequently developed subcutaneously in thiourea-benzpyrene-treated animals were transplanted, at least once, into normal animals of the same strain. Since most of these proved to be sarcomas of local origin, no attempt was made to investigate their properties further.

RESULTS

MORTALITY AND GROSS EFFECTS

The high mortality revealed in Table I provides an index to the toxicity of the dosages of goitrogens given. The use of these toxic dosages was calculated to give the maximum thyroidal effect. The greatest mortality occurred in the initial phases of the experiment. Relatively few animals died as the length of the period of dietary treatment increased. The A strain mice were much more susceptible to the toxic effects of thiourea than the C57 strain. In the dosages given, thiourea was more toxic than thiouracil. Perhaps because of the unpalatability of the diet, surviving mice were very cannibalistic in the presence of a dead or dying mouse. For this reason, often it was not possible

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[1] Thiouracil was generously supplied by Lederle Laboratories and acetyl-aminofluorene, through the courtesy of Mr. T. B. Wallace, by Smith, Kline and French Laboratories.

to autopsy animals dead for more than a few hours. Table I shows, for example, that only 58 per cent of A strain mice started on the thiourea diet were autopsied. The percentage of animals reaching autopsy in other phases of the experiment was somewhat higher. Although tissues of mice dead for an unknown length of time before autopsy often were sectioned, they were not used as the basis for any descriptions of histological changes presented in this report.

Body weights of experimental animals on goitrogenic diets were always lower than controls. Animals on thiourea diets weighed about 12 gm. at 2 years of age in contrast to the normal range of 27 to 30 gm. Body weight plateaued early, or even decreased from a higher initial weight. The body weight of thiouracil-fed mice was less markedly affected. Skeletal growth continued, at least for a time, so that such animals always had a gaunt, emaciated aspect. Hair was very fine and sparse, resembling that of young animals in texture, but not in amount.

It is interesting that the vaginal membranes of females kept on thiourea for as long as 82 weeks had never become perforated. The usual time of vaginal opening in these strains is about 6 weeks. This was not true of thiouracil-fed animals, which, in fact, continued to have litters for about 6 weeks after beginning of their dietary treatment. After this, breeding activity ceased, but no gonadal atrophy was observed.

At autopsy, gross findings were, as a rule, quite uniform. Thyroids were always enlarged. In advanced stages of treatment with either of the 2 goitrogens extremes were seen in which the thyroid extended into the mediastinum below the clavicle. After about 200 days of treatment, large fluid-filled cysts, some as large as 1.0 to 1.5 mm. in diameter, were grossly visible. Great vascularity, and increase in diameter of veins especially, was characteristic. Additional treatment with benzpyrene or acetoaminofluorene had no apparent augmenting effect.

Pituitaries showed little if any gross response. Lungs of all goitrogen-fed animals had frequent congested areas, rarely seen in controls. These appeared as red spots on the surface, about 2 mm. in diameter. Abdominal viscera showed nothing unusual, except for the gonads and accessory structures. In thiourea-fed animals, gonads were of very small (usually infantile) size and yellowish color. Uteri, seminal vesicles, and prostate glands were of infantile size. Thiouracil-fed animals, although apparently sterile after 6 weeks of treatment, had gonads of normal, though small, size, and accessory organs were of a similar range of size.

EFFECTS ON MICROSCOPIC STRUCTURE OF THYROID

A. *Control.*—Few descriptions of normal thyroid morphology in the mouse exist in the published literature against which one may gauge experimental variations. Smith and Starkey (36), describe age changes principally in regard to connective tissue, and Van Dyke (40) describes the occurrence of ciliated cells in thyroids of normal mice. The following brief description of thyroid structure in the mouse supplements these observations and deals specifically with mice of the strains used in the experimental work, fed a stock diet of Purina fox chow. A more detailed account of normal thyroid structure is being prepared separately.

In younger mice of all strains studied, up to 4 months of age, thyroids present a rather uniform appearance (Fig. 1). The general aspect is that of a physiologically active gland. There is a comparatively short antero-posterior axis. The follicles are small, usually about 10 cells in a circumferential section, and colloid usually is well scalloped in the neighborhood of the epithelial cells. Follicular tissue of ultimobranchial origin can be recognized by position and by morphological differences. In young animals a recognizable fibrous-tissue septum often exists between the ultimobranchial thyroid follicles and those of median pharyngeal origin. The most characteristic follicles of ultimobranchial origin are

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DESCRIPTION OF FIGURES 1 TO 6

FIG. 1.—Thyroid of untreated C57 strain mouse, three months of age. Note larger follicles in cortical region. Mag. × 95.

FIG. 2.—Thyroid of untreated C57 strain mouse 470 days of age. Follicular epithelium is irregular and follicles are separated by hyaline type connective tissue. Mag. × 95.

FIG. 3.—Thyroid of A strain mouse after seven days feeding with thiourea. Cells are hypertrophied; colloid is vacuolated. Mag. × 190.

FIG. 4.—Thyroid of C57 strain mouse after 39 days feeding with thiourea. Increased vascularity and cellular hypertrophy; colloid is almost absent; follicular lumina are

irregular due to papillary projections of epithelium. Mag. × 190.

FIG. 5.—Thyroid of A strain mouse after 196 days feeding with thiourea showing growth of cortical follicles and beginning cyst formation. Note papillary and follicular growth into lumina of enlarged follicles, and reaccumulation of colloid in enlarged follicles. Mag. × 95.

FIG. 6.—Thyroid of A strain mouse after 261 days feeding thiourea. The lumen of the medium sized cyst (0.6 mm. long) is partly filled by ingrowing tissue. Parenchyma of very small follicles and cellular cords. Mag. × 95.
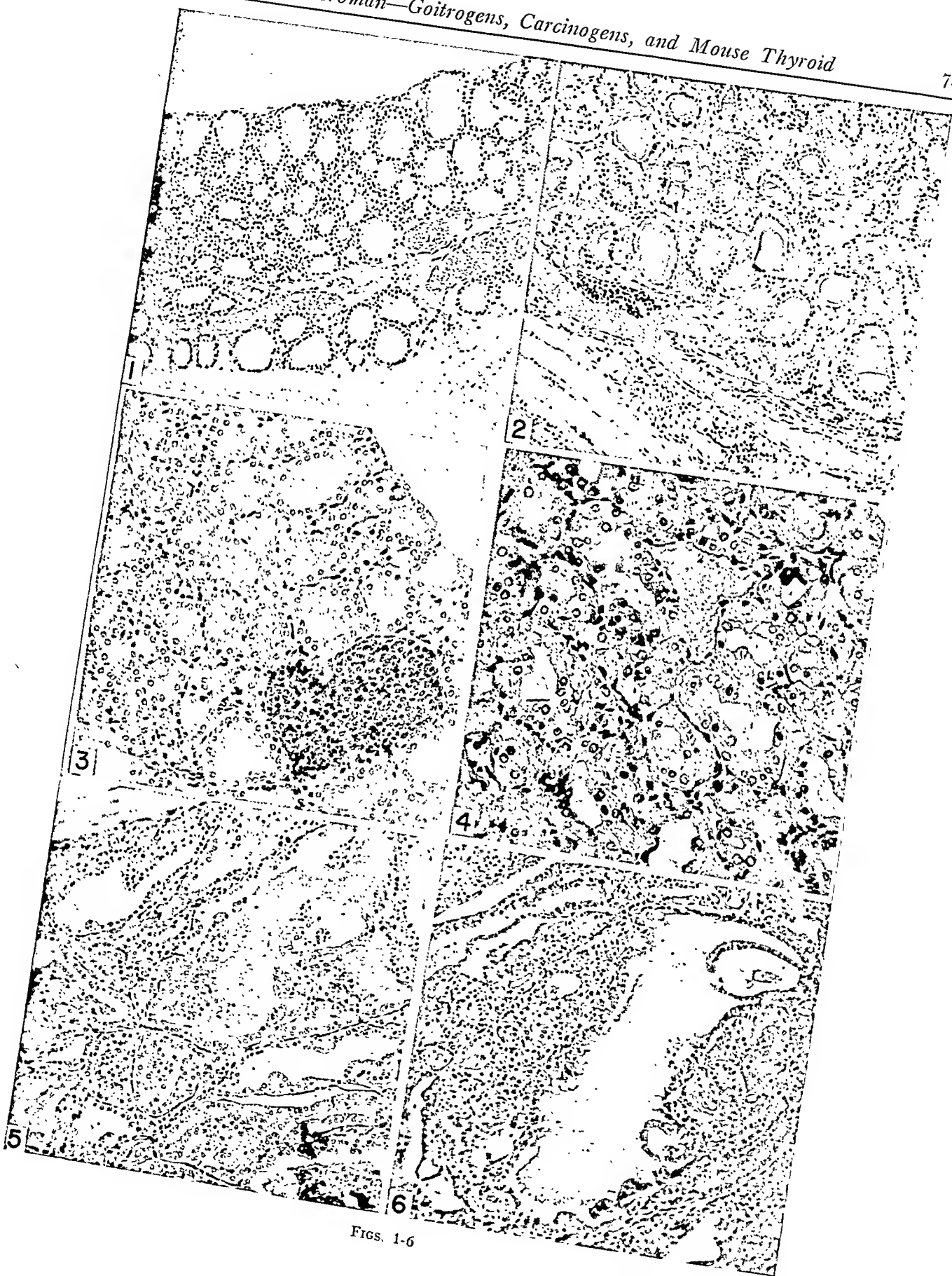
FIGS. 1-6

irregular, often tubular, in shape. They are lined by an alternately cuboidal and squamous epithelium (sometimes stratified). Frequently some of the cuboidal cells are ciliated. Ciliated cells are much more common, however, in thyroids of older animals. Colloid in these ciliated follicles has a very foamy character and is lightly eosinophil in staining capacity. Grouped around such primary ultimobranchial follicles are follicles of apparently derived nature. These satellites of the primary ultimobranchial follicle have a lower epithelium (often squamous) than other follicles, and a denser, unvacuolated colloid. Further removed from the primary ultimobranchial follicle the satellite follicles are increasingly difficult to distinguish from other follicles and merge with them.

In older animals follicles are somewhat larger and an increasingly greater heteromorphism develops between thyroid follicles of lateral and median pharyngeal origin. Normal thyroid follicles are lined by a cuboidal epithelium. Epithelial cells have a fine granular, lightly staining purplish cytoplasm. Colloid is relatively densely eosinophilic and unvacuolated. Primary ultimobranchial follicles in older animals become quite large and complexly branched and stand out clearly from surrounding tissue. Ciliated cells occur in tufts and may be quite prominent. The tendency for development of large ciliated follicles is much greater in the A and I strains than in the C57 strain. Satellite follicles are of two sizes, one smaller and the other larger than surrounding typical follicles. In both types the epithelium is lower and more densely basophilic and the colloid is darker than in other follicles. The larger colloid-filled follicles often form a distinct annular zone around the primary ultimobranchial follicles. There is a progressive infiltration of aging thyroids by fatty and fibrous connective tissue (Fig. 2) until follicles become widely separated.

B. *Initial effects of thiourea and thiouracil.*— The only notable difference in effect on thyroid of the two goitrogens tested (in dosages given) was a slightly more rapid progression of changes on the thiourea diet. For this reason no attempt will be made to distinguish between the effects of these agents. The first effect of thiourea and thiouracil, noted in animals fed for 7 days, is hypertrophy of epithelial cells and reduction in colloid (Fig. 3). Cells are enlarged in width as well as height and encroach upon and decrease the diameter of the follicular lumen. Colloid is highly vacuolated and less eosinophilic. Recognizable ultimobranchial follicles are only slightly affected at this time.

Treatment for 40 to 60 days.—Such hyperplasia continues, advancing in degree, until 40 to 60 days of treatment, when changes in character of the follicles begin. At one or more points in the follicular wall cells become stratified forming local thicknesses (Fig. 4) and finally begin to project into the lumen as low, broad papillae. Epithelial cells at this time are low columnar in shape but considerably larger than in untreated mice. The cytoplasm is uniformly lightly basophilic. The nucleus is large, vesicular, and clear, with most of the visible chromatin near the nuclear membrane. Colloid is almost entirely gone, and is present as occasional lightly eosinophilic strands. There is a striking uniformity in the appearance of the thyroid as a whole, the characteristic differences between the cortical and medullary regions of the thyroid disappearing. This tendency toward uniformity is seen in the ultimobranchial region of the thyroid as well. The distinction between ultimobranchial follicles and others becomes increasingly difficult to make. In almost all animals, however, the primary ultimobranchial follicle with its ciliated epithelium and bubbly colloid still can be found.

Vascularity is increased very greatly. The gross effect of this increase upon examining the thyroid under low power is an apparent separation of the follicular by interstitial elements.

Treatment for 100 to 110 days.—The development of a zone of somewhat larger peripheral follicles by the one-hundredth day of thiourea or thiouracil-feeding eradicates the uniformity in cross-sectional appearance seen in shorter periods of treatment. All follicles at this time are very irregular in shape and contain several papillary ingrowths which vary from broad truncate to narrow pedunculate in appearance. The papillae are composed either of epithelial tissue, or else contain a core of fibrous and vascular tissues.

A striking increase in heteromorphism in epithelial cells occurs at this stage of treatment, in contrast with the previous homogeneity. Especially near the apices of the follicular papillary ingrowths a new small, dark type of cell may be found. Nuclei in such cells are conspicuously smaller though still vesicular. At the apices of papillae some pycnotic cells also may be found. It is possible that this pycnosis in cells so situated is induced by the gradual displacement from their blood supply. In larger cells, forming the major epithelial part of the thyroid, a conspicuous non-granular perinuclear zone develops. Colloid, which was almost completely absent in thyroids of mice treated for 40 to 60 days, makes its reappearance after 100 days. It

appears as a very lightly eosinophilic material which fills the follicles.

The ultimobranchial zone of the thyroid is almost indistinguishable from the remainder of the gland. Follicles in this zone are smaller, however, and contain fewer papillae. In only 1 animal of this group was a ciliated primary ultimobranchial follicle found.

Further vascular changes include: (a) fibrous thickening of the media in larger arteries (*e. g.,* superior thyroid artery), (b) development of venous sinuses, especially near the medial surface, apparently by fusion and consolidation of venous and capillary channels at certain points, and (c) development of small basophilic thrombus-like structures projecting into the lumina of veins in or near the thyroid.

Treatment for 140 to 150 days.—After 140 days of goitrogen feeding an interesting feature is the formation of secondary follicles within pre-existing enlarged follicles. These characteristically develop by accumulation of colloid just beneath the apical epithelium of an intrafollicular papilla. Since this further removes the apical cells from their vascular supply they undergo more extreme pycnotic and even degenerative changes. The free (apical) portion of such secondary follicles becomes extremely thin, apparently stretched by the accumulation of colloid. This colloid would seem to be secreted by the active-looking cuboidal and columnar cells forming the part of the wall of the secondary follicle attached to the primary follicle.

Another feature which is obvious in thyroids at this stage of treatment is a growing disparity in the sizes of follicles. A few unusually large follicles, with a large number of papillary ingrowths are found, most often peripherally. Between them are numerous follicles so small that lumina are difficult to find in any one cross-section.

Characteristic of this stage of goitrogen administration is the gradual accumulation of much interfollicular epithelial tissue. Such interfollicular cells are obviously derived from the walls of larger follicles, and are arranged as very tiny follicles containing a small amount of colloid, or else as non-follicular masses. Two new cytological characters are (a) the appearance of large spherical yellow granules in the cytoplasm of the epithelium of only certain larger follicles, otherwise not different from follicles not showing this feature, (b) the development of enlarged cells whose cytoplasm is almost completely agranular and chromophobic. These empty-looking cells are restricted to the extreme edge of the thyroid (Fig. 8).

In the ultimobranchial region of the thyroid at this stage, no morphological characteristics can be found to differentiate it from the remainder of the gland, and therefore ultimobranchial tissue as such is no longer identifiable.

Treatment for 180 to 200 days.—After 150 days of feeding goitrogen the thyroid loses its regularity of outline, and grossly exhibits a "lumpy" outline. The lateral dimension of the whole thyroid is increased, and there is the appearance of turgidity and pressure against the capsular membrane.

After 180 days, a number of large cystic follicles develop (usually about 10 to 30) which are easily visible even grossly. In size they may be as much as 1.5 mm. in diameter. Microscopically these cystic follicles are characterized by a low cuboidal, or even squamous epithelium, and large numbers of ingrowing papillae (Figs. 6, 7). The epithelium covering the papillae is much more active in morphologic appearance, being composed of large cuboidal or columnar cells. The lumen of the follicular cysts contains also as many as 4 or 5 secondary follicles, whose epithelium is characteristically squamous, and often degenerating. In a few instances a secondary follicle contains a tertiary one. Arranged in ring-like zones about the cystic follicles, and apparently derived from them by centripetal proliferation, are very small follicles and non-follicular epithelial cells. Excepting the cysts, very little colloid-containing space remains in the thyroid, giving the gland at this time a compactly cellular aspect. In a few of the smaller cystic follicles the colloid has a variegated appearance containing irregular dark-staining areas. In the epithelial cells of such follicles yellow pigment granules are found almost always.

Treatment for 220 to 300 days.—The large cysts so conspicuous at 200 days are almost completely filled by 300 days of treatment. The areas made up of cells which have filled the individual cysts have a nodular appearance. The only conspicuous colloid remaining at 300 days is in the few constricted lumina of former cysts.

The cells forming the thyroidal parenchyma are larger, both in size of the nucleus and volume of cytoplasm, and are much more uniform in various parts of the gland. The enlarged non-granular cells found at the periphery of the thyroid in earlier stages of treatment are almost entirely absent by 300 days. The arrangement of cells, especially in the nodules derived by filling of cysts, is as tiny follicles, many without lumina, and in cellular cords. Together with the hypertrophic appearance of the cells, this gives a fetal aspect to the micro-

scopic structure of portions of the thyroid (Figs. 10, 11, 12). The vascular fusions and thrombus formations noted in earlier phases of treatment become cumulatively more extensive.

Treatment for 300 to 500 days.—The longest treatment period was 566 days. However, after about 350 to 400 days when maximum vascular effects seemed to be achieved, there was no further notable structural change.

The progressively greater anaplastic and fetal appearance seen developing earlier is characteristic after 300 days. This is all the more remarkable when it is noted that thyroids of untreated control animals of corresponding age are undergoing senile involution at this time. In thyroids of older mice it has been noted by several earlier observers (1, 36) that a progressive hyaline fibrosis occurs. No indication of such fibrotic changes can be seen in goitrogen-fed mice despite their age, and despite the fact that senile changes are occurring in other organs (*e. g.*, calcification of tracheal cartilages.)

Few of the follicular cysts remain after 300 days, and those which are distinguished as cysts have their lumina almost completely filled by papillary and small follicular cellular groups. Colloid is found only as thinly staining strands, except in the lumina of the former cysts, where it stains strongly with eosin, and fills all of the available space.

It is interesting that in spite of all the growth undergone by the thyroid during goitrogen treatment no obvious mitotic stimulation could be observed. After 300 days, however, mitotic figures are more frequent, an average of 2 being found to each microscopic field under high-dry magnification.

Evidence of pressure within the thyroid may be adduced from several facts. First, it may be seen especially around the cysts that follicles have been squeezed to such an extent that their lumina are greatly restricted and become slit-like. This results in a striking aligning of most cellular elements about the cyst into rows which parallel the circumference of the cyst. Another evidence of mechanical tension is the stretching of the gland capsule, and its rupture at several points (Fig. 9). The penetration of thyroidal tissue through and beyond the fibrous capsule is interpreted as a pressure-induced phenomenon since cells here have as low a mitotic index as elsewhere in the gland.

The most remarkable changes at this stage are in the vascular channels. Especially at the median edge, fusion of veins produces very large continuous blood spaces which may be called sinuses. Because of vascular fusions around them, thyroid follicular masses of all sizes project into these blood sinuses and are separated from them only by the endothelium (Figs. 13 to 16). The thyroid follicles projecting into blood spaces may have a narrow or broad stalk connecting them to the remainder of the thyroid tissue. In a number of instances, verified by careful study of serial sections, wholly free intravascular thyroid follicles were found with no observable parenchymal stalk or connection. In two instances small clusters of cells were found in small extra-thyroidal veins, and in all observable cytological features they were similar to the epithelial cells of the thyroid.

The finding of intravascular thyroid tissue led to a search for possible thyroid tissue in lungs of long-treated animals. Since the advisability of this search became apparent at a time when only a few animals remained in the experiment, lungs of only 22 goitrogen- and goitrogen plus carcinogen-treated animals were studied for this purpose. Eleven controls of comparable age were examined also for any kind of pulmonary growth. In one control nodules of bronchogenic carcinoma were found. In 7 treated animals, all of the A strain, small pulmonary growths were found which were not bronchogenic carcinoma. These growths were cytologically very similar to the hyperplastic thyroid tissue (Fig. 18). They contained small follicular groups of cells which enclosed small amounts of pale colloid-like material. There is much inferential evidence that these are thyroid tissue carried to the lung via the thyroid veins, but this point

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DESCRIPTION OF FIGURES 7 TO 12

Fig. 7.—Thyroid of A strain mouse after 261 days feeding thiourea. and 230 days after injecting 1.0 milligram of benzpyrene subcutaneously. Approximately the same as Fig. 6. Mag. × 95.

Fig. 8.—Same animal as in Fig. 7. Edge of thyroid showing large agranular cell region. Note irregularity of colloid distribution. Mag. × 190.

Fig. 9.—Penetration of thyroid capsule by thyroid (upper right) into striated muscle (lower left). Fibrous capsule runs diagonally through figure. Break in capsule is at lower right corner of figure. Mag. × 190.

Fig. 10.—Thyroid of A strain mouse after 261 days feeding thiourea. Region of filled-in cystic follicles, showing cord-like arrangements of cells. Small amounts of colloid in larger follicular lumina only. Mag. × 95.

Fig. 11.—Thyroid of C57 strain mouse after 466 days feeding thiourea. Cells arranged as small follicles or solid cord-like masses. Colloid is irregularly distributed and heterochromatic. Mag. × 190.

Fig. 12.—Same animal as in Fig. 11. Note the intimate relation of groups of epithelial cells to enlarged vascular channels. Mag. × 95.
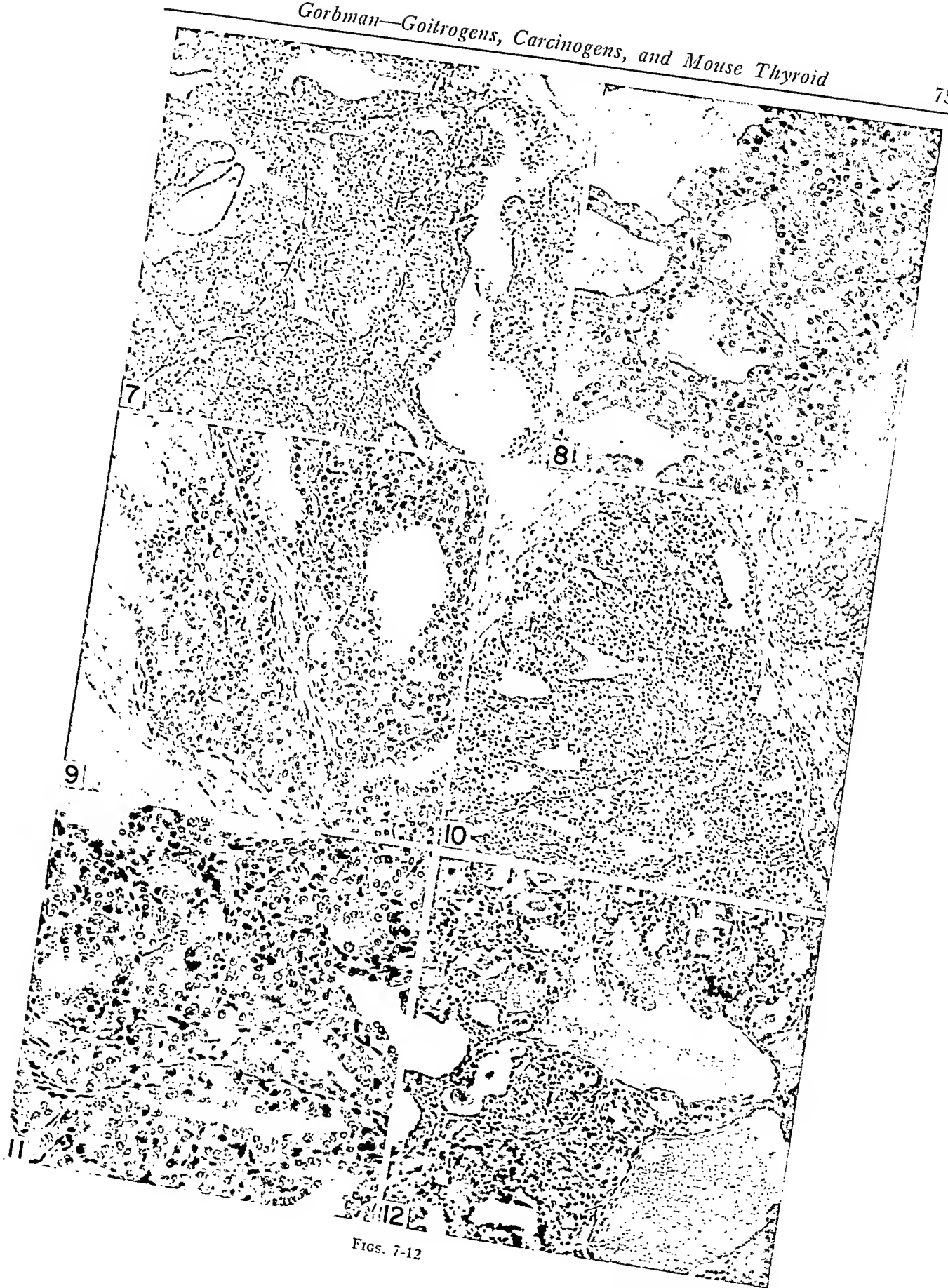
FIGS. 7-12
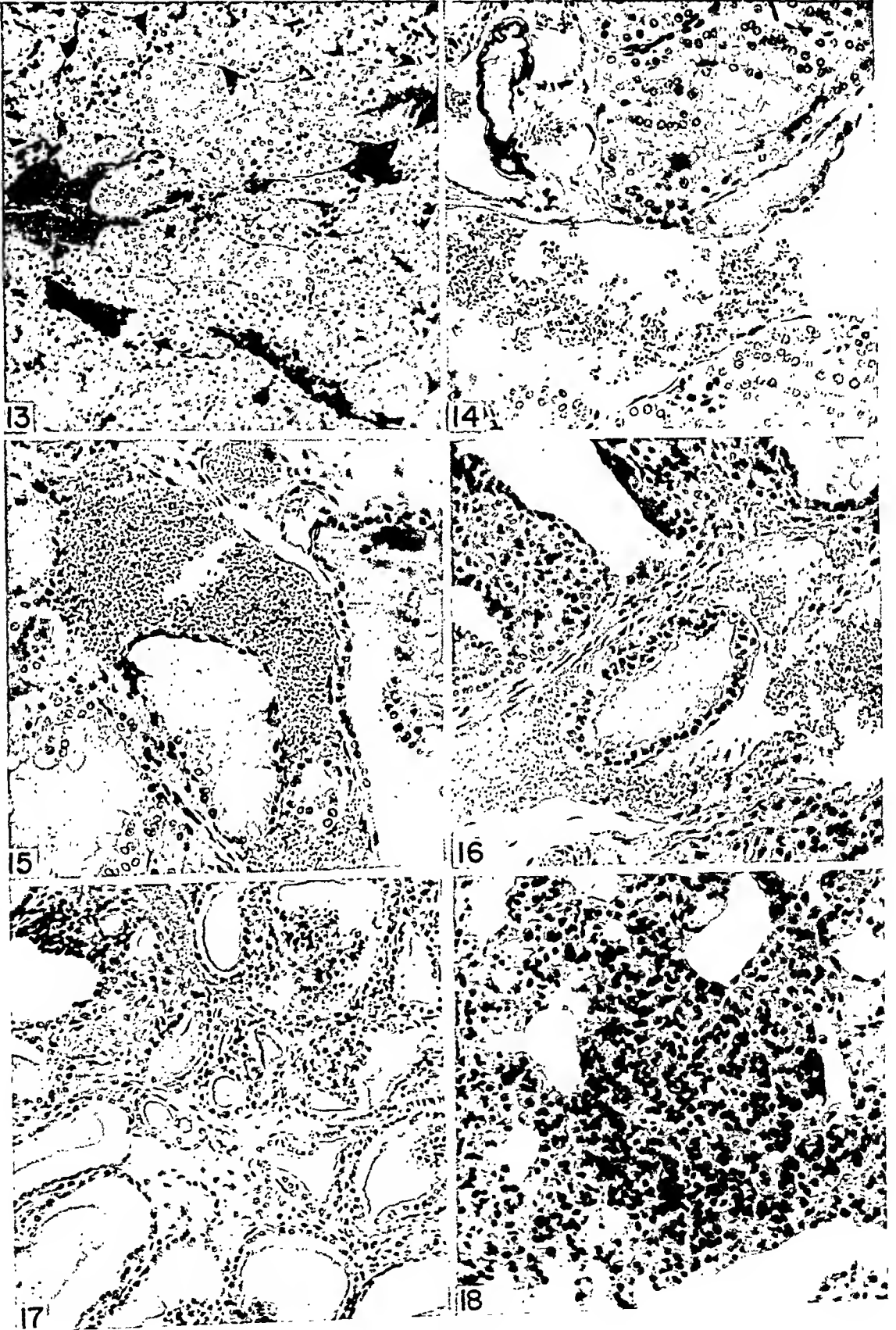
awaits further study with better technics, such as the use of radioactive iodine.

Effect of return to normal diet after 300 to 450 days of treatment.—The most obvious overt effect of returning animals to the normal diet after a period of treatment sufficient to produce very extensive effects is a rapid gain in body weight and improvement in general appearance. The average gain in weight in a period of 12 days of resumed normal diet is 7 gm. Some mice, especially the A strain, experienced relatively little weight gain. This appeared to be due to difficulty in feeding upon solid food pellets after a diet of powdered food for more than one and one-half years. Teeth in such animals are very thin and frail.

Damage to the ovary is not repaired in this interval (only female animals were examined in this phase of the experiment) and the uterus, likewise, remains in a completely inactive condition macroscopically and microscopically.

There is a significant, but not great, reduction in size of the thyroid (average weight 34 mgm.) in 12 days and it is much paler in color. The most striking microscopic change in the thyroid is a decrease in size of the cells and shrinkage of their nuclei. Epithelial cells are all small, cuboidal in shape and contain a much reduced amount of darkly staining cytoplasm. Colloid is present in every follicular lumen. It is solid, unvacuolated, even at the edge, and densely eosinophilic in staining reaction.

The highly developed vascular structure characteristic of long goitrogen treatment undergoes rapid involution. Very few of the capillary and sinusoidal-sized vessels persist between follicles. The larger vessels remain, but leukocytic infiltration of walls of some arteries is evident. A loose type of fibrous tissue occupies interfollicular spaces created by shrinkage of other structures.

Occurrence of tumors in carcinogen-treated mice.—It seems remarkable that no tumors of any kind were found in mice receiving carcinogen (benzyprene or aceto-aminofluorene) as well as the goitrogenic diet *after* 200 days of age. Eighteen locally induced tumors were found in A and C57 strain mice (12 in A and 6 in C57) between 90 and 130 days after subcutaneous injection of 1 mgm. of benzpyrene. This represents 30 per cent of the A strain mice and 30 per cent of C57 mice so treated. None of the mice given aceto-aminofluorene developed tumors of any kind. The benzpyrene-induced tumors were mostly localized sarcomas, and a few were squamous carcinomas. It is significant that in other work (unpublished) the author has induced almost 100 per cent incidence of tumors in A strain mice with benzpyrene. In this strain spontaneous tumors, especially mammary and pulmonary carcinomas, are very frequent. It would appear justifiable to conclude that the goitrogenic treatment suppressed the carcinogenic properties of concomitantly administered benzpyrene and aceto-aminofluorene.

RESUMÉ AND CONCLUSIONS

Both Bielschowsky (3) and Purves and Griesbach (28, 29), who fed rats thiourea for as long as 2 years consider the thyroidal growths obtained to be carcinomatous. In the present experiments with mice, in which treatment was extended for as long as 566 days this conclusion does not seem to be justified. The opinions of the previous authors were based upon morphological grounds and principally upon the appearance of "invasiveness toward the capsule," "invasion of veins," and appearance of thyroid nodules in the lung. Although these same morphologic arguments could be drawn from the present work, the involution following cessation of treatment would seem to invalidate

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DESCRIPTION OF FIGURES 13 TO 18

Fig. 13.—Thyroid of A strain mouse after 261 days feeding thiourea. Fusion of interfollicular vascular channels tends to isolate individual follicles. Mag. × 95.

Fig. 14.—Thyroid of A strain mouse after 261 days feeding thiourea. Follicle covered by endothelium, projects into enlarged venous sinus near right edge of figure. In upper left part of figure, a thrombus-like mass, possibly a degenerate follicle, projects into another blood vessel. Such thrombi are quite characteristic of later stages of treatment and some are quite large. Mag. × 190.

Fig. 15.—Thyroid of A strain mouse after 315 days feeding thiourea. A follicle covered by endothelium projects into a vein. The cells in the middle of the vein are low and pycnotic. The cells in contact with the thyroid parenchyma are very active in appearance. Mag. × 190.

Fig. 16.—Thyroid of C57 strain mouse after 320 days feeding thiourea. A follicle covered by endothelium, projects into a blood vessel, and is attached to the blood vessel wall by a fibrous peduncle. Mag. × 190.

Fig. 17.—Thyroid of A strain mouse after 410 days feeding thiourea, followed by twelve days on normal diet. There is in this time interval a prompt involution of the hypertrophied cells and vascular development, reappearance of dense colloid, and filling of interfollicular spaces with loose fibrous tissue. This gives an appearance of senility similar to that illustrated in Fig. 2. Mag. × 190.

Fig. 18.—Lung of A strain mouse after 450 days feeding thiourea. The nodule contains cells cytologically similar to hypertrophied thyroid. Cells are arranged as small follicles, a few of which (upper right portion of nodule) contain "colloid." Mag. × 190.

these signs of malignancy. Purves and Griesbach (29) have shown that the thyroid "carcinoma" observed in their rats depends upon a continued supply of thyrotropic hormone for maintenance.

Penetration of the thyroidal capsule in mice fed goitrogens (Fig. 9) is a common occurrence, but has no appearance of malignant infiltration. Ample histological evidence is at hand from mice to indicate that such penetration is due to stretching, thinning and mechanical breaking of the glandular capsule. The finding of intravascular thyroidal follicles, and pulmonary nodules of thyroidal tissue would seem to provide more convincing evidence of malignancy. Study of the vascular changes in the thyroid during prolonged treatment shows that precipitation of thyroidal tissue into veins is a passive process and appears in no way due to "invasiveness" on the part of the glandular tissue. Successively more extensive fusion of the capillary and sinusoidal channels about a given follicle, especially at the edge of a venous sinus, isolates it and finally causes it to project into the sinus. Such intravascular, mechanically isolated follicular structures, even when freed of neighboring stromal and epithelial tissue remain completely invested by endothelium. This fact is considered as important in concluding that they have reached their intravascular position without active penetration of the blood vessel wall.

The pulmonary growths found here cannot be classed as metastatic, since their attainment of the intravascular position is a result of activity of the vascular tissue and is not an expression of potentialities of the migrating tissue itself. It is interesting, however, that in these experiments a property of cancerous growths has been imitated and the mechanism by which this is accomplished is explicable. Thorek (37) speaks of clinical instances of extra-thyroidal establishment of thyroid tissue apparently of a benign nature.

The morphological picture after prolonged treatment seems to differ between rats and mice. In rats Bielschowsky (3) first showed that chronic goitrogenic treatment produces a nodular hyperplasia and this growth pattern has been confirmed by others (14, 28, 29). In mice the overgrowth is quite uniform throughout the gland. In early stages of treatment in mice (about 200 days) large cysts are produced, and the filling of lumina of individual cysts by papillary and follicular hyperplasia produces a temporary nodular aspect, similar to that reported in the rat's thyroid. Further treatment in mice (400 to 500 days) produces thyroids whose cells are arranged in fetal patterns, in tiny follicles with or without lumina, and in cords.

Observation of mitotic figures in hypertrophied thyroids was rare, indicating that cellular multiplication contributed very slowly to the growth of the enlarged glands. The largest gland recorded weighed 54 mgm., a ten-fold increase over the average 3 month old control. It is difficult to compare this with very old untreated control mice whose thyroid weights are extremely variable. Thyroids weighing up to 15 to 18 mgm. are not uncommon. Such enlarged thyroids in old mice contain very large amounts of a mucoid connective tissue (Fig. 2). It is of great interest that the thyroidal stimulation, presumably thyrotropic, experienced by goitrogen-treated mice completely allayed these senile changes and preserved the morphological aspect of a youthful cellular vigor. Several authors (1, 2, 36) have shown that connective tissue infiltration is a normal consequence of aging in the thyroids of mice. It seems significant that in 12 days after cessation of goitrogenic stimulation, the large interfollicular spaces created by shrinkage of epithelial and vascular elements are quickly filled in by loose connective tissue. Such involuting glands weigh relatively little less than stimulated ones (average 34 mgm. compared to an average of 48 mgm. in mice under treatment for 400 days).

It has been demonstrated amply by a number of investigators that goitrogenic stimulation of the thyroid is mediated by the hypophyseal thyrotropic hormone. That this mechanism can be maintained in continuous activity for almost 2 years is a significant datum for several related fields. Pathologists have long believed that the thyroid is subject to exhaustion after prolonged stimulation (*e.g.*, the "Marine cycle"). The present evidence appears to require a different explanation in cases of cyclic involution of the thyroid. The continuous effectiveness of thyrotropic hormone for so long a period also would seem to have significance in considering the problem of thyrotropic antihormones.

No evidence could be found to indicate that carcinogens in any way augmented the effect of goitrogens upon the thyroid. On the contrary, it was quite clear that the goitrogens had an anti-carcinogenic effect. Despite the high spontaneous tumor incidence in A strain mice, during goitrogenic treatment such mice had no spontaneous tumors, even in old age. Very few local tumors could be induced in them by benzpyrene, none by aceto-aminofluorene. It is interesting that the same changes in rats' thyroids produced by thiourea and aceto-aminofluorene in Bielschowsky's work (3, 4), were produced by rape seed alone by Griesbach, Kennedy, and Purves (14). Together with the present data such results suggest that the carcinogen has

little or no part in the genesis of the observed thyroidal growths.

Engle and Aronow (9) have drawn attention to the colloid-containing secondary follicles which appear within larger cystic follicles in thyroids of monkeys chronically fed thiouracil. Although these have been found as herein described, in the mouse, similar structures have not been described in goitrogen fed rats. Secondary follicles in mice arise by accumulation of colloid at a point beneath the primary follicular epithelium. This accumulation of colloid takes place actively at a time when colloid resorption is apparent in all other parts of the gland. Such isolated colloidal accumulation in secondary follicles and larger follicular cysts implies a physiological difference in the secretory epithelium involved, if not in the colloid itself. This interesting possibility deserves further study, especially in the light of recent emphasis upon the relation of ultimobranchial tissue to nodular hyperplastic tissue in the rat (40).

It seems quite clear that within the limits of observed variation, the thyroid responses to goitrogen and carcinogen administration are quite uniform in the different pure strains and hybrid strains studied. This is all the more remarkable when it is realized that strain differences in normal thyroidal structure (unpublished data) exist before beginning of treatment.

SUMMARY

Thyroidal changes were studied in mice of the A. C57, and I strains, and in several strain hybrids, during thiourea or thiouracil feeding for as long as 566 days. The carcinogens 3,4-benzpyrene or acetoaminofluorene were given some groups, in addition to the goitrogens, with no alteration in the succession of observed changes.

The most rapid change observed during the first 40 to 60 days is an initial epithelial hypertrophy and exhaustion of colloid. This is followed by a prolonged period of interstitial cellular, follicular, and papillary hyperplasia, which because of the infrequency of mitotic figures. is assumed to be slow. After 150 days localized subepithelial accumulations of colloid in certain thyroid follicles, produce secondary follicles which project into the larger lumen. After 180 days some very large cystic follicles are found. These are gradually filled by papillary ingrowths. After 300 to 400 days the thyroidal epithelial cells assume cytological characteristics and spatial arrangements similar to those of fetal glands. Cells are arranged as tiny follicles with or without small lumina. and as cords.

After 200 days of treatment extensive vascular

fusions produce sinus-like spaces of venous blood. Fusion of capillaries around follicles isolates them until they project into vascular channels covered only by endothelium. In several instances entirely free intravascular masses of thyroidal cells were found. Of a group of 22 mice whose lungs were serially sectioned after more than 300 days' treatment, seven had pulmonary nodules of thyroid-like tissue.

Return to the normal diet after 300 to 450 days goitrogenic treatment produced prompt involution of the anaplastic-appearing epithelium, and a return of dense colloid.

The problems of (a) the role of embryonic ultimobranchial tissue in the observed growths, (b) holding in abeyance of senile involutional changes, (c) localized recurrence of colloid after long treatment and (d) the benign properties of the observed growths are considered in the discussion of results.

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Cytology of Spontaneous Adenomas in the Pituitary Gland of the Rat*

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In recent years, the spontaneous appearance of adenomas in the anterior hypophyses of rats has been described by Wolfe, Bryan, and Wright (39), by Oberling and his associates (24), by Saxton (28) and by Saxton and Graham (29). These adenomatous growths have been found almost entirely in the anterior lobes of old rats and their presence is seemingly a manifestation of advancing age.

The size, appearance and cellular constituents of these adenomatous lesions is variable, Wolfe, Bryan and Wright (39) having described three types. The first type was characterized by varying amounts of hemorrhage and was classified as a hemorrhagic adenoma. These growths ranged in size from small lesions of 0.4 × 0.5 mm., which could not be recognized grossly, to large swollen tumors involving practically the entire anterior lobe. Histologically they were composed of chromophobes although small numbers of acidophiles were present in some instances. The chromophobe cells in these lesions generally, but not invariably, showed various degrees of hypertrophy, some being of enormous size. The adenomatous acidophiles were invariably enlarged. Capsules were not present about these lesions although the neighboring anterior lobe tissue was often compressed.

The second type of lesion was designated as an adenomatous nodule. They were made up of small diffuse masses of enlarged chromophobes or acidophiles and sometimes both. The smallest of these nodules contained only a few cells while the largest measured only 0.4 × 0.6 mm. Hemorrhages were not present and there was no compression of the surrounding tissue.

The third type of adenoma described by these authors was large, occupying practically the entire anterior lobe, and was made up entirely of chromophobic cells of varying size. There was no evidence of hemorrhage.

In all three types of lesions, there was a marked tendency for the nuclei and nucleoli of the adenomatous cells to be enlarged and often there was hypertrophy of the negative image of the Golgi apparatus. Many of the adenomas contained mitoses; in contrast, mitotic figures were rare or absent in the non-adenomatous tissue.

The lesions described by Saxton (28) and later by Saxton and Graham (29) appear to be quite similar to those described above except that adenomatous acidophiles were not described. In many of their animals the lesions were multiple. In preparations stained with Sudan III many adenomatous cells were found to contain fat which was not present in the normal anterior lobe cells. These authors also made intraocular transplants of adenomatous and non-adenomatous anterior lobe tissue. On the basis of the behavior of the transplants of the adenomatous tissue, it was concluded that these growths represented true neoplasms.

Anterior lobe adenomas have now been described in old rats, both male and female, of several strains, and although the data are still rather scanty, it seems clear, as Saxton and Graham (29) have already pointed out, that there are both strain and sex differences in the incidence of these tumors.

The studies mentioned above have furnished considerable information concerning the general histologic structure of these tumors. The purpose of the present communication is to supplement our previous description of these lesions, placing special emphasis on certain cytological aspects not considered previously. In addition, two spontaneously occurring adenomas of the intermediate lobe of the pituitary gland of the rat are described.

MATERIAL AND METHODS

These observations are based on the study of 44 anterior lobe and 2 intermediate lobe adenomas found in the pituitary glands of 24 rats of the Vanderbilt strain and 7 from the Albany strain. The incidence of anterior lobe lesions in old female rats of these strains is 29 per cent in Vanderbilt breeding females (39), 27 per cent in Vanderbilt non-breeding females (36) and 11 per cent in Albany non-breeding females (41). No data are yet available on the incidence of these lesions in the breeding females of the Albany strain.

At autopsy the hypophyses were usually divided into two sagittal halves, fixed in Regaud's fluid, cut serially at 3 microns and stained by the basic fuchsin mitochondrial technic of Fain and Wolfe (13).

Fairly often, however, the glands were preserved in 2 fixatives, one half being fixed in Regaud's fluid and the second half in Champy's fluid. The Champy-fixed tissue was postchromated according to the modified Champy-Kull method of Severinghaus (31) and stained by the mitochondrial method mentioned above. With both Regaud- and Champy-fixed tissue this technic stains mitochondria a brilliant fuchsin, acidophilic granules pinkish, orange or orange-red and the basophilic granules or secretion deep blue. Connective tissue fibers stain blue and red blood cells red or orange red.

OBSERVATIONS

Cytological Studies of Spontaneously Occurring Anterior Lobe Adenomas

During the course of this study, we have observed the three types of adenomatous lesions in the anterior lobe described by us previously; *i. e.*, hemorrhagic adenomas, adenomatous nodules, and chromophobic adenomas (39). Although the three separate types of lesions were easily differentiated from each other, the cytologic appearance of the adenomatous cells constituting the various tumors was similar, *i. e.*, the characteristics of the cells present in adenomatous nodules, in hemorrhagic adenomas and in chromophobe adenomas were generally the same. Therefore, the descriptions of certain adenomatous cells in one type of lesion are usually applicable to the cells of the other varieties.

The drawings of cells shown in Figs. 1 to 27[1] will, therefore, be used to illustrate similar cells found in all types of lesions.

Hemorrhagic adenomas.—Eleven hemorrhagic adenomas were found. These varied in size from a small lesion measuring 0.8 × 0.8 mm. to a large adenoma involving practically all of the anterior lobe. In most instances the normal anterior lobe tissue surrounding the tumors was compressed. Eight of the lesions were purely chromophobic, the remainder contained, in addition to chromophobes, a few acidophiles. As we have pointed out previously (39) these adenomas were characterized by variable amounts of hemorrhage. Scattered throughout the tumors were blood-filled spaces of variable size which apparently did not possess an endothelial lining, their contents appearing to be in direct contact with the adenomatous anterior lobe cells. These interstitial hemorrhages have been more fully described previously (39).

Generally, the adenomatous chromophobes in these tumors were made up of cells showing varying degrees of hypertrophy but it should be emphasized that many adenomatous chromophobes were not increased in size. Numerous cells showed only slight hypertrophy whereas in others the hypertrophy was pronounced; cells measuring approximately 30 × 30 microns were regularly observed

[1] These cells were drawn by Miss Alice Pauline Schafer.

DESCRIPTION OF FIGURES 1 TO 15

All cells are shown at a magnification of × 1440. The acidophilic granules are shown by small circles. Mitochondria are shown in various shades of black which in some cells shade into dark gray. The granular cytoplasm of the anterior lobe basophile and the intermediate lobe cell are grayish. All cells are from the anterior lobe except those specially designated.

Fig. 1.—A small acidophile from the normal portion of the anterior lobe.

Fig. 2.—A moderately, enlarged adenomatous acidophile. Note the very large nucleolus and nucleolar vacuoles. The Golgi apparatus was quite hypertrophied.

Fig. 3.—A much enlarged adenomatous acidophile. There were abundant fine mitochondria and hypertrophy of the Golgi apparatus. The nucleus showed evidence of lobulation.

Fig. 4.—An enlarged adenomatous chromophobe. The large nucleolus contained vacuoles. The mitochondria were quite variable in size and some were vesicular in nature.

Fig. 5.—A small chromophobe from the normal portion of the anterior lobe.

Fig. 6.—A small chromophobe from the normal portion of the anterior lobe. Note enlarged Golgi apparatus and abundant mitochondria.

Fig. 7.—A small adenomatous chromophobe. Note the large nucleolus, the nucleolar vacuoles and the nucleolar protrusion. The Golgi apparatus was much enlarged.

Fig. 8.—A chromophobe of moderate size from the normal portion of the anterior lobe. The Golgi apparatus was enlarged and there were abundant mitochondria.

Fig. 9.—Granular basophile from the normal portion of the anterior lobe.

Fig. 10.—Nongranular basophile from the normal portion of the anterior lobe.

Fig. 11.—Large adenomatous chromophobe with many mitochondria, a few of which were quite large.

Fig. 12.—Large adenomatous chromophobe which measured approximately 55 × 35 microns. The nucleus measured 30 × 20 microns and the largest nucleolus 6 × 6 microns. Note the nucleolar vacuoles and the invagination of the cytoplasm into the nucleus. The mitochondria were variable in size and in the intensity of their staining reaction.

Fig. 13.—A normal intermediate lobe cell.

Fig. 14.—A moderately enlarged adenomatous intermediate lobe cell which had dense blue cytoplasm. It is thought that the scattered canals represented a diffuse Golgi apparatus.

Fig. 15.—A very large adenomatous intermediate lobe cell. The mitochondria were large and vesicular, the center portions took a light fuchsin and the outer borders a darker fuchsin stain. The very large nucleolus contained many vacuoles.

FIGS. 1-15

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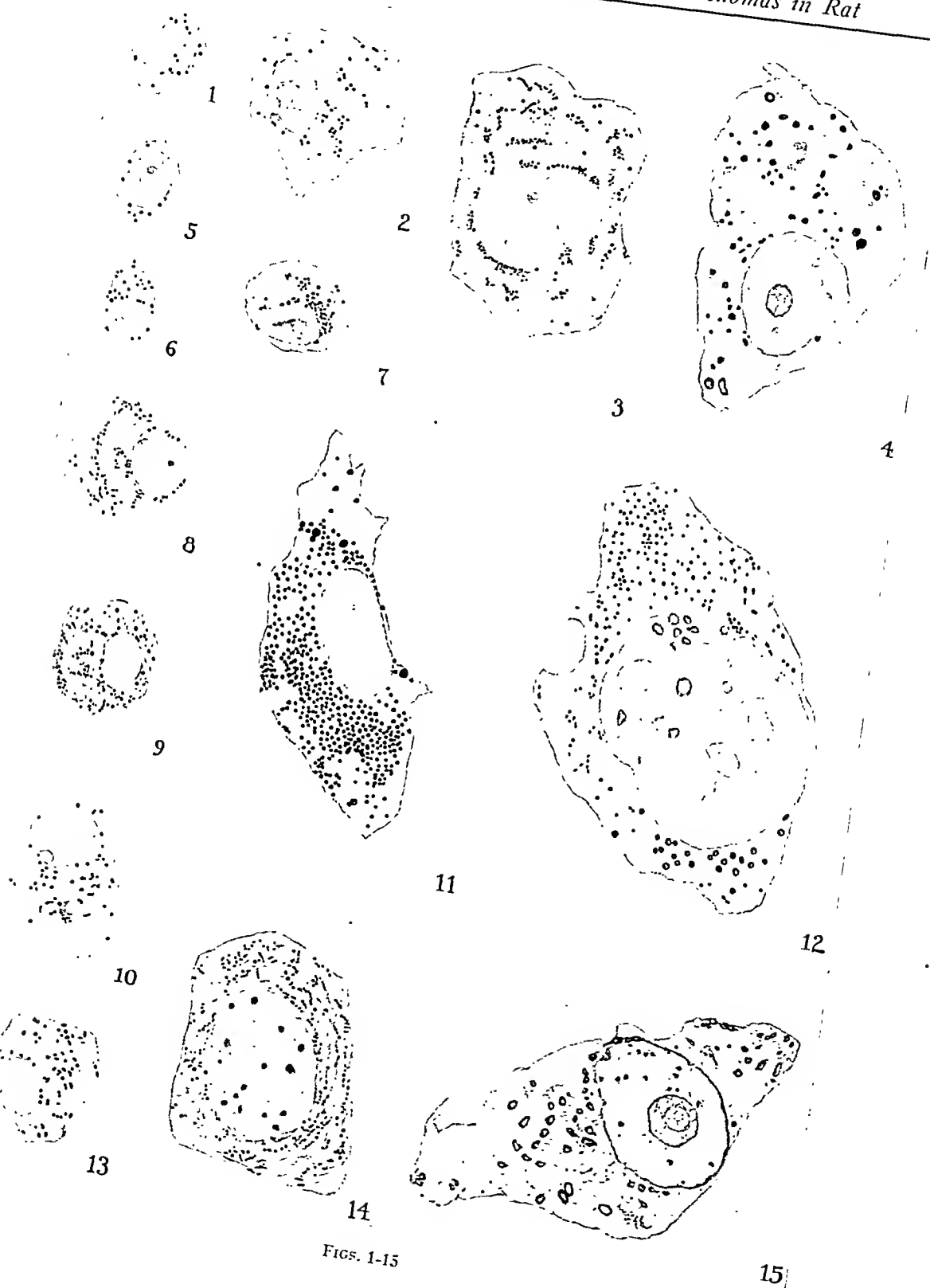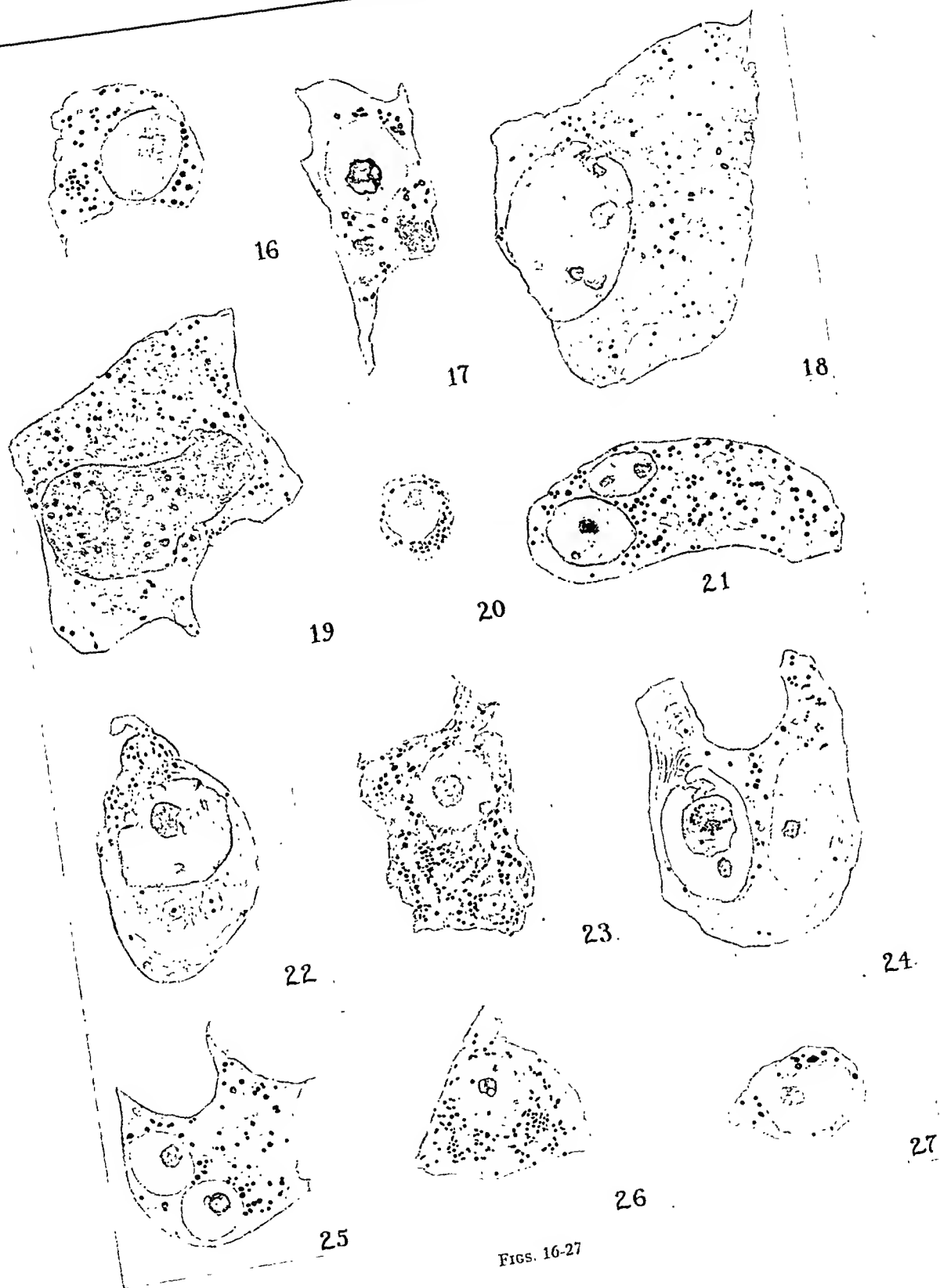
FIGS. 16-27

and cells as large as 50 × 40 microns were seen fairly frequently (compare the normal and adenomatous chromophobes in Figs. 1 to 27). The nuclei and nucleoli were also often but not invariably enlarged, nuclei measuring up to 20 × 28 microns and nucleoli as large as 5 × 5 microns being observed. In fact, nucleolar hypertrophy and to a lesser degree nuclear enlargement were outstanding features of the adenomatous chromophobes and often occurred in cells in which there was no increase in size of the cell itself.

The nuclei of the adenomatous chromophobes were of unusual interest. They tended to be markedly distorted in shape and often the nuclear membrane was wrinkled and indented (Figs. 12, 18, 22, 24 and 27); in many cells the nuclei were lobulated and in some there was evidence of nuclear budding (Fig. 19). Frequently cells containing 2 or more nuclei were seen (Figs. 21 and 25), indicating the possibility of direct cell division. As a rule the nuclei were stained pale blue and the nucleoplasm was homogenous in appearance. In some instances, however, small irregular bodies, stained a dull blue, were scattered throughout the nucleus (Figs. 4, 19 and 22). The staining reaction of these small bodies was generally similar to that of the nucleoli (see below).

In most of the nuclei, one or two large and prominent nucleoli were seen (Figs. 4, 17, 22 and 23) and often they were more numerous. The nucleoli also showed varying degrees of hypertrophy; in a few instances they reached a diameter of 5 × 5 microns (Fig. 12). Usually, they were

stained varying shades of blue but in some cells they were deep red and not infrequently pale red or grayish-orange in the center with a narrow band of blue on the periphery.

As a rule, the nucleoli were more or less rounded in shape (Figs. 4 and 25) but reference to Figs. 7, 16 and 20 will indicate that more irregular forms were often encountered. Sometimes small protrusions of various shapes extending from the main body of the nucleolus into the surrounding nucleoplasm were observed (Fig. 7). Often on the surface of the nucleolus small spherical bodies, apparently made up of material identical to that of the nucleolus, could be seen. Sometimes these bodies could be shown to be continuous with the nucleolus (Fig. 20); sometimes they appeared separate. In addition to these small bodies, which appeared to be actually continuous with the nucleoli or at least very close to them, other small structures were sometimes found scattered throughout the nucleus (Fig. 22). Their staining reaction was identical to that of the nucleoli. The relation of the nuclear structures mentioned above to each other and to the nucleoli is vague and should be the subject of further study.[2]

[2] The staining technic used in this study did not differentiate the nucleoli from the adjoining nucleolus-associated chromatin which has been described in many cells (Hyden, H. Protein metabolism in the nerve cell during growth and function. Acta. Physiol. Scandinav., 6 (Suppl.): 1-136. 1943; Caspersson, T. The relations between nucleic acid and protein synthesis. Symposium Soc. Exper. Biol., No. 1. 1947, pp. 127-143). In recent studies (38) made on nonadenomatous anterior lobe cells of the rat, one or more

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DESCRIPTION OF FIGURES 16 TO 27

All cells showed at a magnification of × 1440. Mitochondria are shown in various shades of black which in some cells shade into dark gray.

Fig. 16.—A slightly enlarged adenomatous chromophobe. The nucleolar mass appeared to be made up of several nucleoli closely packed together.

Fig. 17.—A moderately enlarged adenomatous chromophobe containing 3 large droplets of lipoid (grayish masses surrounded by light areas). The generally large mitochondria in this cell took a pale fuchsin stain. Note the large nucleoli and nucleolar vacuoles.

Fig. 18.—A large adenomatous chromophobe with scant numbers of mitochondria, some of which stained very lightly. The nucleus showed a wrinkled membrane and some evidence of budding.

Fig. 19.—A large adenomatous chromophobe with a large nucleus, a small nucleolus and many smaller bodies which stained identically to the nucleolus. Similar staining but smaller bodies were found in the cytoplasm. Only scant numbers of mitochondria were present.

Fig. 20.—A small adenomatous chromophobe. Note the relatively large nucleolus, the large nucleolar protrusion and the abundant mitochondria.

Fig. 21.—A moderately large binucleated adenomatous chromophobe.

Fig. 22.—Adenomatous chromophobe with cytoplasmic filaments and two nebenkern. Note the practical absence of mitochondria in the region of the filaments. The nuclear membrane was considerably wrinkled, the nucleolus large and vacuolated.

Fig. 23.—Adenomatous chromophobes which had deep blue cytoplasm and a large vacuolated nucleolus.

Fig. 24.—Large binucleated adenomatous chromophobe that showed an evagination of cytoplasm containing mitochondria into the nucleus. Note the cytoplasmic filaments and the two possible nebenkern.

Fig. 25.—Moderately large binucleated adenomatous chromophobe. Some of the mitochondria took a deep fuchsin stain, others were much lighter. Note the large Golgi apparatus.

Fig. 26.—Moderately large adenomatous chromophobe with an enlarged Golgi apparatus and a vacuolated nucleolus.

Fig. 27.—A small adenomatous chromophobe. Note the wrinkled nuclear membrane, the vacuolated nucleolus and the variations in the size of the mitochondria.

We have reported previously (39) that the nucleoli of the adenomatous anterior lobe cells sometimes contained one or more small lightly staining areas or vacuoles. In this study we have encountered such nucleolar vacuoles frequently in adenomatous anterior lobe cells (Figs. 4, 17, 22, 23, 26 and 27). In most of these cells, only 1 or 2 were present but sometimes there were several (Fig. 17). Most often the vacuoles were found well in the interior of the nucleolus (Figs. 12 and 23) but fairly frequently they were on the periphery (Figs. 26 and 27) and sometimes even seemed to lie on its surface.

In those nucleoli which were stained deep blue, the material in the vacuoles was usually pale orange-red in color but sometimes it was merely lighter blue, grayish or colorless. In the nucleoli that stained red, the vacuolar substance assumed a lighter red. In those nucleoli that were stained pale orange-red or grayish-orange in the center and had a deep blue periphery, vacuoles were not observed.

In the normal anterior lobe cells (Figs. 1, 5, 6, 8, 9 and 10), the nucleoli were much smaller than in the adenomatous cells. While they also contained nucleolar vacuoles these were smaller and far less abundant.

The cytoplasm of the enlarged adenomatous chromophobes generally stained a light to moderately deep blue but in many cells it was stained intensely by aniline blue (Figs. 23 and 27). Fairly often, the amount of cytoplasm was small and it appeared fragmented, being pulled away from the nuclear membrane or the cell membrane or both. In most of the hemorrhagic adenomas varying numbers of cells contained rounded cytoplasmic vacuoles of variable size. These are believed to be due to the dissolving out of lipoid droplets which, as will be shown later, can be demonstrated in Champy-fixed tissue.

In most cells mitochondria were moderately numerous (Figs. 7, 16, 21 and 26) but sometimes they were either extremely abundant (Fig. 11) or relatively few in number (Figs. 18, 19, 22 and 24). In shape they were usually spheroidal but sometimes they occurred as short rods, filaments or ovals. In a few cells they were considerably enlarged (Figs. 4 and 27), sometimes measuring up to approximately 2×2 microns. Generally the mitochondria stained a deep red with the basic fuchsin but often they were less fuchsinophilic (Figs. 17, 18, 25 and 27). Very large mitochondria showed a particular tendency to stain less intensely (Figs. 12 and 25). Sometimes the large mitochondria were stained lightly in the center and more densely on the periphery (Fig. 4). Fairly often in this study it has been observed that the cytoplasm in a small circular area immediately around the mitochondria in the adenomatous chromophobes took a lighter blue stain than did the rest of the cytoplasm; this condition is shown in Figs. 16 and 27. This finding suggests that some type of chemical reaction was occurring in the immediate vicinity of such mitochondria which resulted in the altered staining reaction.

In the present report the observations on the Golgi apparatus are limited to a study of its negative image. While we fully realize that such a study cannot furnish precise information concerning its structure, it does give some idea of its size and general shape in the adenomatous cells. In many of the enlarged adenomatous chromophobes the negative image of the Golgi body was markedly hypertrophied, being made up of a system of irregularly anastomosing colorless canals (Figs. 21, 23, 25 and 26). However, in others, the negative image could not be detected, even though serial sections of the cells were studied. Generally speaking, the negative image of the Golgi body was seen more frequently in the slightly or moderately enlarged adenomatous chromophobes, in which it was generally hypertrophied than in the largest cells (compare Figs. 12 and 25).

Since the changes in the Golgi apparatus have generally been correlated with the functional activity of cells, a consideration of this structure in the adenomatous anterior lobe cells is of considerable interest. In several papers (32, 33 and 34) dealing with the cytology of anterior lobe cells, Severinghaus has associated hypertrophy of the Golgi apparatus with elaboration of secretion by the cell and an increase in the number of mitochondria with the release of the secretion. Wolfe and Brown (40) have confirmed the findings of Severinghaus which associated hypertrophy of the Golgi apparatus in the anterior lobe cells with elaboration of secretion and have found a certain amount of evidence which associated an increase in the number of mitochondria with release of secretion from the

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small nodular masses of chromatin were found to be in contact with or close to the nucleolus and in some instances the nucleolus was completely surrounded by a narrow band of chromatin. Both the nodular masses and the perinucleolar chromatin gave a positive test for desoxyribose nucleic acid when stained by the Feulgen technic. We think it quite likely that the small protrusions which extended from the nucleolus and the small bodies lying close to the nucleolus, which we have described in this paper, were actually nucleolus-associated chromatin.

cell. In a later paper Wolfe (37) was unable to associate conclusively an increase of mitochondria with the release of secretion although it was not doubted that an increase of mitochondria indicated increased functional activity of the cell.

Using these cytologic criteria as a basis, our observations indicate that many of the adenomatous chromophobes were in an active secretory state since they showed pronounced enlargement of the Golgi apparatus and contained numerous mitochondria. Since they showed cytologic evidence of the elaboration of secretion and yet no storage of it in the form of resolvable granules, it is considered likely that the secretion was being released into the blood stream as rapidly as it was formed. Although many adenomatous chromophobes did show undoubted cytologic evidence of the formation and release of secretion, our observations do not necessarily indicate that excessive amounts of secretion were being formed and it should be stated that any evidence other than the cytological findings just referred to, that these adenomas were producing and releasing hormones, was not found.

Not infrequently, in some of the most noticeably hypertrophied chromophobes a negative image of the Golgi apparatus was not observed. However, examination of cells such as that shown in figures 4 and 18 suggests that the Golgi material occurred in the form of fragments scattered throughout the cytoplasm. These cells contained many small clear vacuoles and careful microscopic examination revealed that the vacuoles were arranged in a pattern suggestive of a markedly hypertrophied Golgi body in which the material composing it had become quite diffuse. If in some cells the Golgi material became even more diffuse, perhaps being scattered through the cytoplasm in the form of isolated fragments, it seems possible that the negative image of such bodies, which would appear as small vacuoles or clear spaces in the cytoplasm, would be difficult and perhaps impossible to recognize with any degree of certainty. Although we know of no definite instance where the Golgi body of anterior lobe cells has been found in such a diffuse and fragmented state, it does not seem inconceivable that this actually was the case in the adenomatous cells considered here. On the other hand, it might also be possible that in many of these enlarged adenomatous cells, for reasons as yet unknown, the Golgi material was contracted or compressed into such small structures that the negative image was overlooked. It is thus quite clear that a study of the Golgi apparatus by these methods is less satisfactory than by methods that color this body (osmic

or silver impregnation). Only by such a positive method will be obtained a satisfactory knowledge of the structure and behavior of this body in adenomatous cells of the anterior hypophysis.

In many of the hemorrhagic adenomas, the enlarged adenomatous cells were often irregular or unusual in shape (Figs. 17, 23 and 24). The reason for this irregularity is believed to be due to the fact that the adenomatous cells were tightly packed together and were presumably exerting pressure on each other. Certainly, they compressed the normal anterior lobe tissue surrounding them.

As previously noted, 3 of the hemorrhagic adenomas also contained adenomatous acidophiles. These cells showed varying degrees of hypertrophy (Figs. 1-3), the largest found measuring 25×25 microns. The hypertrophied acidophiles exhibited the same nuclear hypertrophy and the same enlargement in size of the nucleoli as were noted in the chromophobes, although the degree of hypertrophy, lobulation, wrinkling of the nuclear membranes and enlargement of the nucleoli were not as prominent. The acidophiles were almost always well filled with granules, the negative image of the Golgi body usually was markedly hypertrophied (Figs. 2 and 3) and mitochondria, while moderately abundant (Figs. 2 and 3), were never pronouncedly so. Since hypertrophy of the Golgi apparatus is usually associated with elaboration of secretion, it would seem that these adenomatous acidophiles had produced large amounts of secretion which was stored in the cells as granules. On the other hand, the stimulus which ordinarily induces the acidophiles to release their secretion into the blood stream was apparently nonoperative; at least there was no cytologic evidence that the adenomatous acidophiles were releasing secretion. In these adenomatous acidophiles, therefore, we encounter a cytologic condition which suggests a imbalance between the processes of the elaboration and the release of secretion; these cells had the capacity to elaborate secretion but not to release it in detectable degree.

In one of the hemorrhagic adenomas, a cytological condition which we have never noted before in anterior lobe cells of any type, was observed. In the enlarged nuclei of a considerable number of hypertrophied chromophobes, round bodies surrounded by a deep blue staining membrane were found. These bodies were made up of a substance that resembled the cytoplasm of the cell and even contained red spherical bodies identical in appearance to mitochondria; in other words, it was as if masses of cytoplasm were being encountered within

the nuclei. A study of serial sections of individual cells showing this phenomenon has led us to conclude that these curious bodies were formed as a result of the inclusion within the nucleus of small polypoid masses of cytoplasm which had formed nuclear invaginations (Figs. 12 and 14). These invaginations were usually enlarged at their terminal end but were reduced in diameter as they approached the nuclear membrane (Figs. 12 and 24). The cause of such cytoplasmic invaginations is, of course, unknown, but it seems possible that the pressure of the anterior lobe cells on each other —and in this adenoma there was definite evidence of such pressure—may have played a role. As stated previously, the nuclei of these adenomatous cells were often wrinkled, indented and lobulated. It seems reasonable to conclude that the same abnormality in the structure of the nuclear membrane which made these conditions possible might also have been responsible in whole or in part, for the cytoplasmic invaginations into the nucleus.

Two hemorrhagic adenomas were of particular interest. Both were fairly large (one measured 1.2 × 1.9 mm. and the other 1.5 × 1.6 mm.). They were made up almost entirely of small, closely packed chromophobes whose nuclei were only slightly enlarged and contained 1 or 2 moderately hypertrophied nucleoli (Fig. 27), which contained variable numbers of nucleolar vacuoles. These cells had scant amounts of rather dense blue cytoplasm and often the cell membranes were too indefinite to be seen so that the cells resembled a syncytial mass. As a result of the reduced amounts of cytoplasm present in these adenomatous cells, many more nuclei were seen in a single microscopic field than in a field of similar size in the normal portion of the gland. Although numerous and fairly large blood-filled spaces were scattered throughout the tumors, there were large foci of adenomatous tissue where capillaries were not visible, so that many cells seemed far removed from a visible source of blood supply. Mitochondria were usually quite abundant in these small adenomatous cells, and in many the negative image of the Golgi apparatus was visible and often moderately enlarged. In one of these adenomas a few fairly large chromophobes, measuring up to 40 × 50 microns with nuclei of 25 × 15 microns and nucleoli 4 × 4 microns, were found scattered among the smaller cells. The outstanding structural characteristics of these adenomas, however, were not cell hypertrophy but a slight and fairly constant nuclear enlargement and a more definite nucleolar hypertrophy.

As mentioned previously, Saxton (28) has recently reported that cells in anterior adenomas in rats contain fat while normal cells do not. Our findings confirm his observation. In this study anterior lobe tissue containing 6 of the hemorrhagic adenomas were fixed in Champy's fluid for 24 hours. In 4 of these lesions variable numbers of the adenomatous cells contained brownish-yellow droplets (Fig. 17), which varied in size from small structures not much larger than mitochondria to larger bodies measuring as much as 10 × 10 microns. In some cells only 1 or 2 of these droplets were seen; in other cells they were much more numerous and often occupied the greater part of the cytoplasm. These inclusions we have presumed to be lipoid in nature. The fact that they were not stained black we think was due to the fact that they were subjected only to the relatively small amounts of osmic acid in the Champy's fluid for only 24 hours. That they were lipoid in nature seems to be evidenced also by the fact that they were identical in appearance to inclusions found in the pituicytes observed in the same sections in which there happened to be some posterior lobe present. Gersh (16) has demonstrated that the inclusions in such posterior lobe cells are lipoidal in nature. The lipoid droplets were found chiefly in the adenomatous chromophobes but sometimes they were also seen in the greatly hypertrophied acidophiles. In some instances small lipoid droplets have been observed in occasional cells of the normal portions of the gland, and in 1 or 2 cases they were quite numerous. The significance of the lipoid inclusions in either the adenomatous or normal cells is unknown but it seems obvious that their abundance in many of the adenomatous cells must be considered indicative of an altered cellular metabolism.

In 3 of the hemorrhagic adenomas cytoplasmic filaments were found in many of the chromophobes (Figs. 22 and 24). Sometimes only 1 or 2 were seen in a cell; other cells were nearly filled with them (Fig. 22). Often the filaments occurred in the form of rounded masses (Fig. 22), forming the structures known as "nebenkern." Cytoplasmic filaments and nebenkern have been described in several varieties of cells previously (3) and in the anterior lobe they are in no sense restricted to adenomatous cells since they have been described by Kirkman (18) in the normal anterior hypophysis of the guinea pig, by Desclin (12), in the chromophobe cells of the rat hypophysis during pregnancy. lactation and after estrone administration and by Wolfe and Brown (40) in the anterior lobe of the

rat after injections of estrogen. The significance of these structures is not known. By many they have been regarded as fixation artefacts (3) and our experience would support this viewpoint, since in the present study they have been found much more frequently in Champy-fixed tissue than in that fixed in Regaud's fluid. In the normal anterior lobe, they have been observed most often in chromophobes in glands in an active secretory state, *i.e.*, during pregnancy, lactation and treatment with estrogen. Presumably under these conditions the cytoplasm of the chromophobes is in a physical or chemical state which makes the formation of the structures possible. The fact that we found similar cytoplasmic filaments and nebenkern in adenomatous cells would suggest that the cytoplasm of these cells was in a somewhat similar state. These structures were not found in the normal chromophobes of the glands used in this study. However, it should be pointed out that most of these cells were small and inactive.

In 6 (approximately 55 per cent) of the hemorrhagic adenomas numerous mitotic figures were found; in the normal tissue of only 1 gland a single mitosis was observed in a chromophobe.

Adenomatous nodules.—Twenty-seven adenomatous nodules were examined. These ranged in size from groups of only a few cells to masses measuring 0.9 × 0.9 mm. in diameter. Twenty nodules contained only chromophobes, 5 contained both chromophobes and acidophiles, while 2 were composed only of acidophiles. These nodules were generally diffuse, the adenomatous cells and the normal cells of the surrounding tissue intermingling freely at the periphery of the lesion. There was no compression of the surrounding normal anterior lobe cells and no microscopic evidence of hemorrhage.

Most of the nodules were made up of chromophobes showing varying degrees of hypertrophy although in a few instances the adenomatous cells were quite small (see various normal and adenomatous chromophobes already referred to in Figs. 1-27). In the lesions containing enlarged chromophobes, the largest cells measured up to 50 × 50 microns in diameter while cells measuring 30 × 30 microns in diameter were common. Nuclei and nucleoli were also enlarged, the former measuring up to 10 × 15 microns and the latter to 5 × 5 microns in diameter. The nucleoli often contained vacuoles. The nuclei in the enlarged chromophobes resembled those already described in the hemorrhagic adenomas.

The adenomatous acidophiles varied in size from cells slightly larger than normal to those measuring

approximately 30 × 30 microns, with nuclei measuring up to 10 × 15 microns and nucleoli 3 × 3 microns. Generally, the negative image of the Golgi apparatus was markedly enlarged and the cells were well filled with granules. Mitochondria were moderately abundant (see Figs. 1, 2 and 3).

Four adenomatous nodules were of unusual interest. They were made up entirely of small chromophobes (Figs. 7 and 20), no larger in size than many of the chromophobes in the non-adenomatous regions of the gland. The cells had scanty light blue cytoplasm, were closely packed together, and often had undiscernible boundaries. In many cells, mitochondria were abundant; in others they were not. In the majority of the cells the negative image of the Golgi apparatus could not be seen but in some it was observed. The nuclei of the vast majority of the cells were normal in size although in a few cells they were slightly enlarged. The nucleoli, however, were definitely enlarged and were more numerous than usual. They were stained deep blue and were usually more or less rounded but in some cells they were drawn out on one side and appeared club shaped. In many instances they contained vacuoles. The outstanding and only constant structural abnormalities found in these four lesions, therefore, were the changes in the nucleoli. Considering our material as a whole, we are inclined to the view that as anterior lobe cells undergo adenomatous changes, the alterations in the size and appearance of the nucleoli are among the first discernible changes.

Mitoses were found in only 4, or approximately 15 per cent, of the 27 adenomatous nodules observed, a much lower percentage than was found in the hemorrhagic lesions. Only 2 hypophyses containing adenomatous nodules had been fixed in Champy's fluid and in each of these, adenomatous chromophobes containing lipoid droplets were found.

Chromophobe adenomas.—Four of the adenomatous lesions were classified as chromophobe adenomas. The smallest of these measured 0.9 × 1 mm. while the largest occupied practically the entire anterior lobe. These growths were usually well demarcated from the surrounding anterior lobe tissue and in most instances there was an actual compression of the bordering anterior lobe cells. With one exception they were made up of chromophobes which showed varying degrees of hypertrophy and were in every way similar to those already described in the hemorrhagic adenomas and the adenomatous nodules. No further description therefore seems necessary except to point out that

the 2 of these lesions which had been fixed in Champy's fluid were found to have lipoid droplets in the adenomatous cells. Mitotic figures were present in 3 (75 per cent) of the growths; none were present in the surrounding normal tissue.

CYTOLOGIC STUDIES OF SPONTANEOUSLY OCCURRING INTERMEDIATE LOBE ADENOMAS

In 2 glands adenomas of the intermediate lobe were found. One of these lesions measured 0.4 × 0.6 mm. and was well demarcated from the adjoining anterior and posterior lobes. The other was roughly conical in shape, its apex being in the dorsal region of the intermediate lobe and its larger base in the ventral portion; its greatest diameter was 1 × 1.3 mm. As the lesion extended ventrally it encroached on the anterior lobe, causing slight compression of these cells.

Both these adenomas were made up of intermediate lobe cells which, together with their nuclei and nucleoli, showed varying degrees of hypertrophy (Figs. 13-15). In the smaller growth the largest cells measured 45 × 30 microns, the largest nuclei 18 × 14 microns and the largest nucleoli 3.5 × 3.5 microns. Between these very large cells and the ordinary intermediate lobe cells, all gradations in size could be found. The smaller adenomatous cells stained deep blue, approximately of the same intensity as the normal intermediate lobe cells. As their size increased, however, there was a noticeable tendency for the adenomatous cells to stain more lightly, although there was considerable variation. Many of them were stained light blue while others were almost colorless. In many of the cells the negative image of the Golgi apparatus was noticeably hypertrophied (Fig. 14) but it was also noticed that often, in the largest cells, the Golgi body could not be seen at all, a situation previously encountered in the anterior lobe tumors. As a rule mitochondria were fairly abundant (Fig. 14); were generally spheroidal in shape, and most often showed no tendency to be hypertrophied. The nuclei, like the cells, showed varying degrees of hypertrophy (Fig. 14). They exhibited, but to a lesser degree, the same type of structural abnormalities found in the nuclei of anterior lobe adenomatous cells. The nucleoli took a moderately intense red stain and in a few instances contained vacuoles. Mitotic figures were found occasionally while none were present in the normal intermediate lobe tissue.

The larger growth differed in certain respects from the smaller one. Cell hypertrophy together with nuclear and nucleolar enlargement were more generalized although the limit of cell size was no greater than in the smaller adenomas. As a whole, the cells took a lighter blue stain, the negative image of the Golgi apparatus was seen less often and the cells contained fewer mitochondria, although in a few cells the mitochondria were hypertrophied and occasionally were vesicular (Fig. 15). The nucleoli were generally stained red and nucleolar vacuoles were quite abundant (Fig. 15). The cells in this larger intermediate lobe adenoma often contained cytoplasmic filaments or fibrillae, although nebenkern were not observed. Mitotic figures were present but were not found in the adjoining normal intermediate lobe tissue.

DISCUSSION

This study raises several questions that seem worthy of discussion. First, what is the relation of the various types of lesions in the anterior lobe to each other? Do the hemorrhagic and the chromophobe adenomas arise as such or do they result from structural modifications in an earlier lesion, such as the adenomatous nodule? The present findings seem to support the second possibility. Although there was some overlap in size between the largest adenomatous nodules and smaller lesions of the two other varieties, the former were generally much more minute and involved a far smaller number of cells. No hemorrhagic or chromophobe adenomas as small as the smaller adenomatous nodules were found.

Considering the structural characteristics of the lesions, it seems to us that the two larger types developed as a result of progressive changes in the smaller adenomatous nodules. In the case of the hemorrhagic adenomas, it is likely that these changes included dilatation of the sinusoids and associated hemorrhage, a more definite demarcation from the surrounding normal anterior lobe cells, and often actual compression of these cells. These two latter changes could be expected to occur as a result of the growth of the adenomatous nodule. Presumably the chromophobe adenomas developed in the same fashion except that hemorrhage was absent. It appears equally clear that hemorrhagic adenomas could also be formed as the result of hemorrhage in pre-existent chromophobe adenomas. If we accept the assumption that the hemorrhagic and the chromophobe adenomas were formed as the result of structural changes in the adenomatous nodules, the question arises as to what percentage of the adenomatous nodules actually underwent such modification and what factors were involved. Unfortunately these are questions for which answers are not available.

Second, is there a relationship between the struc-

ture of the various adenomatous lesions and that of the surrounding normal tissue? It has already been mentioned, for example, that many of the adenomatous cells presented cytological evidence of functional activity. Were such adenomatous cells most likely to occur in anterior lobes in which the normal cells also show evidence of secretory activity? Our findings indicate that they were not. Adenomas containing functionally active cells were found in anterior lobes in which the normal cells showed cytologic evidence either of little or no secretory activity, of mild secretory activity, and in only 1 case of pronounced secretory activity. We have reported previously (36) that the anterior lobes of old female rats generally present cytological evidence of decreased secretory activity. Since this is the phase of life in which spontaneous anterior lobe adenomas usually appear there would seem to be some sort of relationship between decreased secretory activity of the anterior lobe cells and the occurrence of adenomatous lesions. The adenomatous cells, therefore, appear to be at least partially independent of the factors that regulate the activities of normal anterior lobe cells. The relative abundance of mitosis in the adenomatous cells and their almost complete absence in the normal cells would point to the same conclusion. Our findings, therefore, support the views of Saxton and Graham (29) who concluded that anterior lobe adenomas were true neoplasms.

During the course of the present study an observation which may be of some significance in relation to the origin of the spontaneously occurring adenomatous lesions was made. In the anterior lobes of old female rats, whether or not tumors were present, we have occasionally observed widely scattered single cells, usually chromophobes, which, when compared with the surrounding cells, contained slightly but definitely hypertrophied nuclei and nucleoli. If such cells had been multiple, forming small clusters or clumps, the clusters thus formed certainly would have been classified as adenomatous nodules. Whether these scattered individual cells actually were of the same nature as true adenomatous cells, and really played a role in the genesis of the adenomatous lesions, are matters of both conjecture and interest.

It is informative to compare the cytological characteristics of adenomatous and normal anterior lobe cells. A prominent feature of the former was their frequent enlargement. However, the degree of hypertrophy was extremely variable and many adenomatous cells were not enlarged at all. Indeed, sometimes adenomatous chromophobes were actually smaller than the surrounding normal chromophobes. Thus, although adenomatous cells tended to be larger than normal cells, enlargement was not constant.

The nuclei of the adenomatous cells were also generally enlarged but the degree varied greatly. In some lesions the enlargement was slight or absent and in practically every adenoma, cells in which the nuclei were not enlarged at all could be found. Other changes noted fairly frequently in the adenomatous cells were nuclear lobulation, nuclear budding, wrinkling of the nuclear membrane, and the presence of multiple nuclei. With the exception of occasional wrinkling of the nuclear membrane such changes were not found in the non-adenomatous cells. A few adenomatous cells also showed cytoplasmic invagination into the nucleus, a phenomenon not observed in normal cells.

Hypertrophy of the nucleoli was probably the outstanding single cytological characteristic of the adenomatous cells although the degree of enlargement was extremely variable. Hypertrophied nucleoli were not present in all cells classified as adenomatous but it is possible that many such cells contained enlarged nucleoli which were not actually detected. In many instances successive serial sections through the enlarged nuclei of adenomatous cells were studied. When this was done there was not much possibility that any nucleoli were overlooked. However, the study of serial sections of single adenomatous cells was not a routine procedure. It is, therefore, possible that many nuclei contained enlarged nucleoli which went unnoticed. Although we feel that increase in size of the nucleoli was perhaps the most constant change noted in the adenomatous cells such enlargement was not invariably found. It should be noted, moreover, that very considerable enlargement of the nucleolus in non-adenomatous anterior lobe cells may be induced by experimental means. It is well established that the anterior lobes of rats which have received sufficient amounts of estrogen contain many chromophobes which show various degrees of enlargement. These cells contain enlarged Golgi bodies and abundant mitochondria (33, 34 and 40) and usually the cytoplasm stains deep blue with aniline blue (38). It has been suggested by Severinghaus (33) and by Wolfe and Brown (40) that these cells are in an active secretory state. Studies which are being carried out in this laboratory indicate that such cells contain nucleoli which are moderately but definitely enlarged when compared with those in smaller chromophobes which do not exhibit cytological evidence of functional activity.

The presence of light staining areas, or nucleolar vacuoles as we have called them, in the nucleoli

of the adenomatous cells was another interesting cytologic feature. Although they were also found in the nucleoli of normal anterior lobe cells they were much larger and many times more numerous in the adenomatous cells.

Changes in the cytoplasm of the adenomatous cells were less conspicuous than those in the nuclei. Lipoid droplets, often large and numerous, were found in variable numbers of the cells. Smaller droplets were found occasionally in normal cells. In many adenomatous cells mitochondria were extremely numerous, much more so than in normal cells, yet such an increase was not constant and in a few of the adenomatous cells relatively few mitochondria were found. Mitochondrial hypertrophy was pronounced in a few of the adenomatous cells but not present at all in normal cells of the glands examined. However, it should be pointed out that mitochondrial hypertrophy may occur in enlarged but non-adenomatous chromophobes of rats which have received estrogen.

Our observations on the Golgi apparatus in this study are admittedly unsatisfactory since only the negative image of this body was observed. In many adenomatous cells, however, hypertrophy of the Golgi apparatus was extremely pronounced, although this finding was by no means constant. In contrast, the Golgi bodies in the cells of the normal portions of the glands were generally small or only slightly enlarged. In nonadenomatous anterior lobe cells of estrogen-injected rats, however, enlargement of the Golgi apparatus is also pronounced (33, 34 and 40). Therefore, hypertrophy of the Golgi apparatus in adenomatous cells of the anterior lobe is not only not a constant, but also not an exclusive, cytological characteristic.

Analysis of these findings indicates that within the limits to which the comparison was carried there was no constant or unique cytoplasmic or nuclear change which differentiated adenomatous from normal anterior lobe cells. The structural differences between the two types of cells generally seemed to be of degree rather than kind. It is interesting to point out that Cowdry in a recent review (8) has described the differences between cancer and normal cells as quantitative rather than qualitative. The same is true when normal and adenomatous anterior lobe cells are compared.

Although we are not dealing with cancerous cells in this consideration of anterior lobe adenomas, it is found that when adenomatous and normal anterior lobe cells are compared, many of the differences between the 2 types are the same as those which have been described as occurring between malignant and normal cells. For instance Ludford

(20) has reported that cancer cells tend to be enlarged, both the nuclei and the cytoplasm being involved. More recently Cowdry and Paletta (9) in an analysis of squamous cell carcinomas induced in mice by methylcholanthrene found that the malignant cells in some growths were larger than the surrounding hyperplastic cells; in others they were smaller. The findings are similar to what we have observed in anterior lobe adenomas since cell hypertrophy was often found but it was not a constant feature.

Many investigators have reported that the nuclei and nucleoli of malignant cells are enlarged; for a review of the extensive literature, see Cowdry (8), von Hamm and Alexander (35), Cowdry and Paletta (9), Biesele and his associates (2) and Caspersson and Santesson (7). MacCarty and his associates have been unusually active in this field. MacCarty, Haumeder and Berkson (23) have reported that both the nuclei and nucleoli of malignant cells are hypertrophied, the degree of increase being greater in the nucleoli. Later MacCarty (22) reported that the much larger size of the nucleolus in relation to the nucleus in malignant cells might be used as a basis for the diagnosis of malignancy, although this criterion has not been generally accepted (7-9 and 35). Cowdry and Paletta (9) in a study of squamous cell carcinoma in mice found that in many instances both the nuclei and nucleoli of the malignant cells were larger than those of the surrounding cells, but in some of the growths they were either of the same size or actually smaller. Because of this inconstancy they concluded that the size of the nuclei, or indeed of the nucleoli or the cell itself, could not be used as a single diagnostic criterion of malignancy.

Although Caspersson and Santesson (7) believe that nucleolar enlargement occurs in practically all tumor cells they do not regard it as indicative of malignancy. They point out that nucleolar hypertrophy may be found in cells of irritated tissues although the degree of enlargement is not usually as great as that found in malignant cells, and that in certain normal cells the nucleolus might sometimes be quite large. Frugoni (14), in a study of pituitary adenomas in the human being, reported that the nuclei and nucleoli were slightly enlarged in adenomas classified as simple, and markedly enlarged in those considered to be active. He associated enlargement of both the nucleus and the nucleolus with cellular activity. In the pituitary adenomas studied by us hypertrophy of the nucleus and nucleolus was often, though not constantly, found but the degree of enlargement was extremely variable and was sometimes not present.

Regan, Page and MacCarty (27) have reported the presence of unstained nucleolar bodies in both malignant and normal cells and have pointed out that such structures are larger and more numerous in the former. In a later and a more detailed study Page, Regan and MacCarty (25) described two types of intranucleolar structures, refractive bodies which occurred as unstained areas or as vacuoles, and argentophil bodies which stained with silver technics. The refractive bodies were larger and more numerous in malignant and benign tumor cells than in normal cells; while the argentophile bodies were more numerous in the neoplastic cells than in the normal. In fact, they concluded that the more malignant the neoplasm the more numerous were the intranucleolar bodies. These authors pointed out that previous to their studies intranucleolar bodies had already been found in many varieties of normal cells by numerous investigators; these studies they reviewed rather fully. It seems clear that the intranucleolar vacuoles observed by us in adenomatous and normal anterior lobe cells are similar to the refractive bodies of Page, Regan and MacCarty and our findings are similar to theirs in the sense that, in our studies, intranucleolar vacuoles are larger and occur more frequently in adenomatous than in normal cells. The significance of these intranucleolar bodies is unknown. Page and his associates (25) have suggested that they are concerned with the changed metabolism of fast growing cancer cells, a view that seems entirely logical. However, since they occur in nonmalignant cells, although to a much lesser extent, it seems that they must represent the visible expression of some phase of nucleolar function in normal as well as in adenomatous cells.

The findings on the cytoplasmic structures of malignant cells have been inconstant. This question has been considered and reviewed by Ludford (20, 21) and Cowdry (8). Although variations from the normal have been described in both mitochondria and Golgi bodies, no constant or characteristic modification of these cell structures have been found in malignant cells. In anterior lobe adenomas there was marked hypertrophy of the Golgi apparatus and in some cells an increase in the number and size of the mitochondria. These changes, however, were inconstant and were in no way characteristic of the adenomatous cells.

Without further consideration of the literature, it seems legitimate to conclude that the adenomatous anterior lobe cells present many of the variations from normal which have been described for cancerous cells. The significance of these findings is difficult to evaluate at the present time.

The significance of the nucleolar changes, both the enlargement and the presence of intranucleolar vacuoles which often occurred in the adenomatous hypophyseal cells, is not clear. However, recent advances in our knowledge of the structure and function of the nucleolus indicate that these changes are worthy of further study. In certain organs of the rat Biesele (1) has found a maximum of 6 nucleoli in cells with a diploid set of chromosomes. Studies made during the last few years indicate that the nucleoli are formed during the telophase by or under the influence of certain chromosomes and that they disappear during the succeeding prophase; see Gates (15) for review of the literature. More specifically, Caspersson and Santesson (7) state that the nucleoli are produced within or close to heterochromatic portions of chromosomes. They believe that nucleoli are products of the activity of the heterochromatin.

The function of the nucleolus is still only vaguely known. Both Ludford (19) and Sayhs (30) found that nucleoli were increased in size in cells whose metabolic activity was high, and several workers state that nucleoli are large in cells concerned with protein synthesis (7, 10, 17). In the last few years our knowledge of the nucleolus has been materially advanced by the studies of numerous investigators, particularly those of Caspersson and his co-workers, whose investigations were concerned with nucleic acids and their relation to protein synthesis. Ribonucleic acid has been shown to be present in the nucleoli, around the nuclear membrane, and in the cytoplasm of many cells, particularly those that are rapidly growing (5). It is quite abundant in the cytoplasm of cells that are producing secretion rich in protein (6, 7). Furthermore, ribonucleic acid is responsible for the cytoplasmic basophilia which is characteristic of such cells (7, 11). Ribonucleic acid seems to be intimately related to the synthesis of protein (4, 6, 7 and 26), and apparently the activities of heterochromatin, nucleoli and the cell membrane are closely associated in this process (7). According to Caspersson and Santesson (7) proteins rich in hexone bases are produced by heterochromatin and these make up the principal portion of the nucleolus. These proteins migrate toward the cell membrane where the production of ribonucleic acid occurs; the latter seems to stimulate the production of cytoplasmic proteins. Therefore, the nucleolus seems to play a definite role in nucleic acid metabolism and protein synthesis. Since the same authors (7) have also demonstrated that the formation of ribonucleic acid and the synthesis of protein are unusually active in certain malignant cells, it is

natural to suggest that the pronounced nucleolar changes noted in many of the adenomatous anterior lobe and intermediate lobe cells might indicate some sort of dysfunction of the metabolism of nucleic acid and proteins. A further study of adenomas with this thought in mind might well yield valuable information.

SUMMARY

Three types of adenomatous lesions were found in the anterior hypophyses of old female rats, *i.e.*, hemorrhagic adenomas, adenomatous nodules and chromophobe adenomas. The first two types of lesions contained chromophobes and in some instances acidophiles also. The last type contained only chromophobes. Although the three separate types of lesions were easily differentiated from each other, the cytological appearance of the cells constituting them were identical.

The adenomatous chromophobes usually, but not invariably, exhibited various degrees of hypertrophy when compared with normal chromophobes. Quite often, however, they were not enlarged at all and rarely they were even smaller than normal. Hypertrophy of the nucleus occurred more frequently than hypertrophy of the cell as a whole while enlargement of the nucleolus was found in a great majority of the adenomatous cells. The degree of enlargement of the adenomatous chromophobes, of their nuclei, and their nucleoli was extremely variable. The enlarged nuclei were often surrounded by wrinkled nuclear membranes, or exhibited lobulation and evidence of nuclear budding. Binucleate cells were frequently found. The nucleoli, in addition to being enlarged, often contained nucleolar vacuoles. Nucleolar vacuoles were also found in the normal chromophobes but much less frequently.

The adenomatous chromophobes often contained hypertrophied Golgi bodies. Often increased numbers of mitochondria were found and occasionally hypertrophy of these bodies occurred. Lipoid droplets were found fairly frequently in the adenomatous chromophobes and infrequently in the normal cells.

The adenomatous acidophiles constantly showed various degrees of hypertrophy. These cells were always well filled with granules. The Golgi apparatus was generally enlarged and mitochondria were generally abundant. The same nuclear and nucleolar changes which occurred in adenomatous chromophobes were also found in the adenomatous acidophiles but usually the changes were not so pronounced.

Mitoses were found frequently in the adenomatous cells; they were practically absent in the normal cells.

The cells found in the two intermediate lobe adenomas were characterized by varying degrees of enlargement. The nuclear changes were generally similar to those found in the anterior lobe lesions. In many of the adenomatous cells the Golgi apparatus was enlarged and often an increase in the numbers of mitochondria was found.

Comparison of adenomatous cells with normal cells in both the anterior and intermediate lobes indicates that adenomatous cells did not possess any distinctive cytological characteristics which would permit their differentiation from normal cells.

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Parasitization of Mouse Sarcoma 180 by Vaccine Virus and Its Effect on Tumor Growth*

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In the design of these experiments the governing idea has been that microorganisms might selectively infect neoplastic cells and then operate competitively to restrict tumor growth. The decision to try first to establish such a relation for some member of the class of filtrable viruses was made because these agents are intracellular and could, therefore, be capable of interference with cell metabolism at fundamental levels perhaps otherwise inaccessible.

Levaditi (5-9), Rivers and Pearce (10-12) and Findlay and MacCallum (2) established that viruses may infect tumors of rodents and, in addition, be carried there for such unusually long periods of time as to suggest that perhaps malignant cells cannot develop an immunity to virus (12). The behavior of the neoplastic cells appears for the most part to have been unaffected. Levaditi observed necrosis of tumor, but evidently only as part of a generalized infection of the host which was usually fatal (5, 7). However, the more recent experience of Andrewes (1) indicates at least the possibility that as the result of the presence of a virus the proliferation of neoplastic cells may be modified in some way without the complication of host injury. He found that an infectious fibroma of rabbits, itself caused by a filtrable virus, can support Virus III. The fibroma ordinarily regressed spontaneously in an average period of 33 days, but the carriage of Virus III was associated with a reduction of the time for this process to about 19 days. The suggestion was made that the change perhaps represented an example of the interference phenomenon.

In these reports little attention has been paid to the amount of virus involved, a consideration that could be supposed to be of importance for any effect produced, whatever its mechanism. Quantitative relationships have, therefore, been examined in the experiments now to be described, in which a particular effort has been made to introduce large amounts of virus into a tumor.

METHODS

Sarcoma 180 was the 180th spontaneous mouse tumor to be studied in the Crocker Institute for Cancer Research, Columbia University: It has now been transplanted for more than 30 years, and has shown exceptionally vigorous growth. Analysis of the results of grafting 21,663 mice over the period 1914 to 1934 has been made by Haagensen and Prime (3). The tumor grew progressively in 98.4 per cent of the animals. "Cure" by ulceration, necrosis, and slough occurred in 1.08 per cent, and spontaneous regression in only 0.33 per cent. Thus, fewer than 1 animal in 20 can be expected to survive inoculation of Sarcoma 180. Moreover, the behavior of this tumor has been remarkably constant from year to year. Appreciable fluctuations in percentage of "takes" and regressions are infrequent, though they may be encountered.

Grafts of sarcoma 180 were made routinely into the subcutaneous tissues of the right flank of mice at about the level of the costal border. The amount implanted was standardized as one piece of about 1.5 mm. in all diameters. The fragments were introduced by a large needle and trocar in the customary way.

The strain of vaccine virus employed was neurotropic. To begin with, a specimen of commercial calf-lymph was mixed with an equal amount of a solution of penicillin and streptomycin (2,500 units of each per cc.) and 0.3 cc. was then injected intracerebrally into a rabbit. Four days later there were signs of encephalitis. The brain was removed, and, having been found bacteriologically sterile, was macerated and suspended in 4 parts of broth.

Inoculations of 0.025 cc. of this suspension were now made into the brains of mice under light ether anesthesia. After a few blind passages, the animals regularly developed fatal encephalitis. For the remainder of the work intracerebral passages in mice were made at frequent intervals.

While adapted to mouse-brain, the virus has been found to have the wide scope of tissue affinities that characterizes most strains of vaccinia. Thus, it retains a dermotropism and produces typical lesions in the skin of the rabbit. It gives rise

to discrete pox on the chorioallantoic membrane of the embryonated hen's egg. For the mouse it is lethal if given intracerebrally. Moderate doses, however, may be given subcutaneously or intravenously without the development of any gross lesions or impairment of health.

For routine tests for virus a piece of the brain, or tumor, or other tissue to be examined, was cut up with scissors in a mortar and macerated thoroughly. A suspension was then made by adding about 4 parts of broth containing per cc. 1,000 units both of penicillin and of streptomycin. It may be noted that this standard antibiotic broth was used routinely as a vehicle throughout these experiments and appeared to influence the results only in providing complete control of bacterial contamination. Titrations were performed by making serial ten-fold dilutions from 0.2 cc. of the 1:5 suspensions. The precaution of changing pipettes for each tube in the titration was observed routinely.

Tests for the presence of virus, were carried out in rabbits. The shaved skin of the back of grey chinchillas weighing 2,500 to 3,500 gms. was marked off into squares and about 25 tests carried out in each animal; 0.05 cc. of suspension was inoculated intradermally and reactions were examined after 48 hours. Final readings were taken on the sixth day. Positive reactions usually appeared between 48 and 72 hours after inoculation.

The mice employed were for the most part of a Swiss strain bred in the Medical Department laboratories for some 15 years and proven to be satisfactory for investigations concerning a number of respiratory viruses. C57 black mice, obtained from the Jackson Memorial Laboratory, Maine, were also used for a few experiments, as indicated below. All mice were between 4 and 8 weeks old at the beginning of each experiment.

Preparation of virus-injected sarcomas.—For the introduction of virus into tumors a major consideration was that the infective dose should be large, and an experiment was designed with this end in view. First, sarcoma was grafted routinely in the flank of several animals. Six days later a certain number of the tumors, which had reached the size of a pea, were injected directly with 0.1 cc. of a 1:5 suspension of vaccinia-injected mouse brain. At the same time an equal number of control tumors were prepared by injection of suspensions of normal mouse brain or of heat-inactivated (100° C.) virus. Four days later both control and infected sarcomas were removed and pieces of them were implanted into new hosts for a study of their comparative development.

RESULTS

The proliferation of vaccine virus in sarcoma 180.—Established grafts of tumor that had been injected on the sixth day of growth with stock 1:5 suspensions of infected mouse brain were tested at intervals thereafter for virus. At 24 hours the virus was recovered only in small quantities, *i.e.*, in titer of 10^{-1} or less. By the second day, however, the titer was found to be as high as 10^{-7}, and it reached a maximum soon after, running out sometimes to 10^{-5} by the fourth to sixth day after injection.

If fragments of sarcoma were exposed *in vitro* to suspensions of vaccinia and then implanted in mice, the virus was taken up and proliferated in the growing tumor. Pieces of untreated sarcoma were prepared as for routine grafting. They were placed in a test tube containing a 1:5 broth suspension of infected mouse brain and allowed to remain there for 2 hours at room temperature. They were then washed several times with normal saline and grafted into 7 mice. Two weeks later the animals were sacrificed. Tumors had grown in 6. Vaccinia was recovered from all of these; for 2 a titration was performed and the titers were found to be 10^{-5}.

Following almost complete inactivation by heat this strain of vaccinia appeared to multiply in sarcoma 180. A 1:5 suspension of infected mouse brain was placed in a 56° water-bath for 2 hours. Mice inoculated intracerebrally with the heated suspension died only after 13 days, and the mortality rate was then 50 per cent. An unheated suspension of this concentration may be expected to kill all mice injected intracerebrally within 4 days. Two sarcomas were injected with the same heated suspension. Four days later the tumors were removed and ground with 4 parts of broth, and 2 mice were inoculated intracerebrally with 0.025 cc. of each preparation. All of the mice died on the third or fourth day. Evidently the virus had been almost completely inactivated by heat and upon intracerebral injection could kill only slowly and irregularly. Passage through the sarcoma, however, rapidly restored high pathogenicity, presumably through growth of virus and local increase of its concentration in the tumor.

Comparative development of grafts of vaccinia-injected and control tumors.—The rate of growth of grafts of tumors prepared as described above has been examined in several ways: (a) by noting at intervals after transplantation the number of animals bearing distinctly palpable nodules, (b) by sacrificing the hosts after some days and weighing the tumors, (c) by an examination of the fatality rate.

In 5 different experiments 99 mice have received vaccinia-infected grafts of sarcoma 180, while parallel controls numbered 106. By the 7th day after implantation 97 (91 per cent), and by the 10th day 100 (94 per cent), of control grafts could be appreciated as distinct nodules giving unequivocal evidence of growth of the transplant. Meanwhile on the seventh day, only 38 (38 per cent) of the infected implants could be felt and on the tenth day this number had increased to no more than 59 (60 per cent). There is thus a pronounced inhibition of virus-infected grafts during the first week or 10 days of growth.

If the tumors were removed and weighed 11 days after implantation, similar findings were encountered. In one such experiment the mean weight of 20 control tumors was 0.77 gms., almost 3 times the mean weight, 0.27 gms., of 18 tumors containing virus. Moreover, while there was considerable variation in weights of individual tumors in both groups, the largest infected tumor (0.5 gm.) weighed less than the mean of the controls (0.77 gm.), and not half as much as the largest single control sarcoma (1.3 gm.). Again, within the first 2 weeks there is evidence of a clear difference in rate of growth between vaccinia-infected and control tumors.

Table I indicates the results of an experiment in which the animals were left for a longer period of

TABLE I: FATALITY RATE IN MICE BEARING SARCOMA 180
EFFECT OF VACCINIA INFECTION OF TUMOR

	Controls	Infected
Number of mice*	30	28
Died of tumor	27 (90%)	16 (57%)
Failure to grow	0	4 (14%)
Regressions	1 (3%)	7 (25%)
"Cure" by slough	2 (7%)	1 (4%)

*C57 strain.

time to submit to the natural course of events. On the tenth day after grafting the first control had died of tumor, and by the end of the third week 90 per cent of these animals had died in the same way. Meanwhile, relative slowness of growth of infected sarcomas was noted during the first week, and was further demonstrated in a delay in the occurrence of deaths of the hosts. Four transplants did not grow at all. Of the 24 (86 per cent) that did grow, no less than 7 regressed, while one was cured by slough. Thus, in all, 43 per cent of the mice survived implantation of virus-infected grafts.

The main point is once more that the growth of the virus-infected tumors was slower and ultimately less successful than that of the controls.

The number of animals is small, yet perhaps sufficient to suggest an increased incidence of regression for infected sarcomas.

The tumor appears less able than normal tissues to destroy or eliminate virus. Perhaps it would not be surprising to find small traces of vaccinia persisting in sarcomas. But large amounts of virus, titering out to 10^{-3} to 10^{-7} were recoverable from many of the tumors, including those removed even in the phase of regression. The sarcoma is thus differentiated from susceptible normal tissues, in which it would be unusual to find virus at all after 2 weeks from the time of invasion.

There is no evidence that the changes observed were the consequence of ill-health of the animals or of generalized vaccinial infection. In all experiments weights were taken every two days. No significant differences from normal controls were encountered. The mice did not look sick, and virus could not be recovered from the viscera of those bearing infected grafts, though in several separate experiments this point was examined at intervals after transplantation.

It might be that a pox virus would be capable of producing inflammation which could interfere with the proliferation of a tumor in the neighborhood. This has not been found. Moreover, attempts to produce subcutaneous lesions by the injection of concentrated suspensions of this strain of vaccinia into the flank have failed. Tumor nodules have been examined microscopically at intervals after grafting, and there is no evidence of inflammation around the infected grafts, either in the connective tissues or the overlying epithelium. It also seems unlikely that there is sufficient alteration in the vascular supply or the bed of the implant to account for the degree of retardation of growth. The detailed histological findings will be presented elsewhere (4).

Only a partial answer can be given to the question as to whether immune mechanisms of the host may contribute to the effect. The inhibition of growth is seen even during the first few days after transplantation and it is not likely that antibodies to the tumor would develop within this time in sufficient quantity to exert such a pronounced action. The influence of active immunity to vaccinia has been tested. Mice were immunized by subcutaneous injection of 0.1 cc. of suspension of vaccinia-infected brain. Two weeks later they withstood intracerebral challenge. In these mice, vaccinia-infected grafts were again found to grow more slowly than control grafts and at about the same rate as in non-immunized hosts. For the

early retardation of growth of vaccinia-infected sarcomas, therefore, no evidence has yet been uncovered to suggest that an immune mechanism is responsible. Analysis of subsequent development of the tumors and of their regression is incomplete, and it may well be that antibodies of some sort contribute appreciably to the changes seen in the later stages of growth.

DISCUSSION

While the findings have been left temporarily incomplete in several respects, including the histological changes, two principal points may be made. First, vaccinia virus can be shown to possess considerable affinity for a malignant tumor and within the tumor it may attain high concentration which can persist for an unusually long time without causing obvious injury to the host. Second, after transplantation the tumor carrying large amounts of virus does not grow as rapidly as it otherwise would.

As for the proliferation of virus in the sarcomas, it seems fair to assume that this occurs within the neoplastic cells rather than in the stroma. A rigid proof can hardly be given in the absence of visible inclusion bodies, and these are not likely to form in cells derived from mesoderm. However, the well-known predilection of viruses for rapidly growing normal tissues, adult and embryonic, makes an infection of vigorously growing tumor cells reasonably probable. Moreover, the virus persisted in the sarcomas for a longer period of time and in higher concentrations than could be expected from simple invasion of the supporting connective tissues.

The retardation of growth of infected tumors has been demonstrated by a method which is not unobjectionable. In the past, the direct injection of various chemical agents into neoplasms has been followed at times by restriction or cessation of growth, yet introduction of the same agents in other ways, *e.g.*, intravenously, has proved quite without effect. The method has been employed here, nevertheless, largely in order to secure concentrations of virus that it may or may not be possible to attain otherwise. Moreover, the behavior of the tumors has been studied after their subsequent transplantation into new hosts, so that an injurious action of the initial injection upon the vascular supply and bed of the implant cannot be held accountable for the results. Therefore, whatever the mechanism involved may be, it probably concerns primarily the neoplastic cells rather than the supporting tissues.

While it seems likely that the inhibition of growth of the tumor is due largely to a direct action of virus on the tumor cells, further detailed analysis of the phenomenon must depend on the accumulation of quantitative data. It would appear that large amounts of virus are necessary for the effect, and it may be that varying concentrations of virus can be shown to exert correspondingly different degrees of growth restriction. Without such evidence the hypothesis of competitive antagonism remains unsupported. Other explanations may be offered, for example, it is possible that the virus elaborates a toxin capable of producing severe damage to cells.

In any case, there are certain aspects of the problem that would appear to deserve further investigation. Among them may be included the possibility of influencing the growth of an established tumor by intravenous injection of virus, and the extension of the observations to other tumors and other viruses.

SUMMARY

A strain of vaccinia that was adapted to mice has been shown to be capable of infecting sarcoma 180. Within the tumor the virus may attain high concentrations (titres 10^7 to 10^8) which can persist for long periods of time without causing obvious injury to the host. Upon transplantation tumors carrying large amounts of virus grow more slowly and less successfully than they otherwise would. Infected grafts have been found to weigh less than half as much as controls after 11 days of growth, and they have been observed to regress more frequently.

The explanation is offered that the virus acts directly on the neoplastic cells. The mechanism of action is not clear, and may involve the operation of a viral toxin or a competitive antagonism between virus and the infected neoplastic cells.

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Studies on the Transmission of Avian Visceral Lymphomatosis
I. Variation in Transmissibility of Naturally Occurring Cases

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INTRODUCTION

The term visceral lymphomatosis has been applied to the condition in chickens in which one or more of the visceral organs contain abnormally large accumulations of lymphoid cells (26). This condition has also been referred to as lymphatic leukosis (13), lymphadenoma (30), lymphocytoma (15), hepato-lymphomatosis (22), hemocytoblastoma (25), lymphosarcoma (36), and has been regarded by many (1, 16, 21, 30, 33) as a malignant neoplastic disease.

Reports of results on attempts to transmit visceral lymphomatosis have either been highly contradictory or inconclusive. Some investigators (18, 30) were unable to transmit this disease by inoculation. Because of negative results obtained, other investigators (15-17) held the view that lymphomatosis is a nontransmissible disease. However, more recent reports (3-5, 22, 35, 36) indicate that lymphomatosis may be transmitted with cell-containing inocula under favorable conditions. Others (25, 28, 29, 32) have expressed the view that the various manifestations of lymphomatosis, erythroblastosis and granuloblastosis are expressions of the same disease process, and result from a common etiologic agent. These interpretations have been based in part on experiments in which lymphomatosis has occurred among the controls, and conclusions were drawn from statistical differences in incidence of the various manifestations between control and inoculated groups. No specific attempts were made to exclude the possibility that the investigators worked with a mixture of agents—a reasonable explanation for the diverse manifestations obtained.

It has been shown repeatedly that erythro- and granuloblastosis can be transmitted readily with filtrates [see reviews of Olson (33), Engelbreth-Holm (14), and Furth (23)], and that the etiologic agent has many characteristics ascribed to viruses (20, 24, 27, 37). Thus, if one accepts the unitarian view (25) one may hypothesize that all forms of leukosis in chickens are produced by a virus-like agent; however, such reasoning has many weaknesses (21, 23) and is of much less significance than evidence obtained from well controlled experiments showing a high rate of, and direct transmission of, a particular form of the avian leukosis complex (26).

In view of the controversial status of visceral lymphomatosis and the lack of conclusive evidence concerning its transmission, experiments were conducted to test the transmissibility of this manifestation. These experiments have been conducted with a uniformity of environment and host which has hitherto not been attained. It has already been reported (5) that, under certain conditions, cell-containing preparations from tumors of some cases of visceral lymphomatosis will induce a high incidence of tumors in a short time; whereas similar preparations from other cases will produce only a few, if any, tumors. Four inocula that elicited a high proportion of tumors were propagated by serial transfer through 15 to 19 passages made at intervals of 7 to 14 days.

Experiments described herein further demonstrate the variation in the transmissibility of visceral lymphomatosis by cellular inocula from different naturally occurring cases. They also show that cell-free preparations from certain cases of visceral lymphomatosis, when injected into chicks, will produce lymphomatous involvement of the viscera and, in some instances, osteopetrosis.

MATERIALS AND METHODS

Donors.—The birds that supplied the material for inoculation were obtained either from the Laboratory flock used in the genetic study of this disease or from a group of birds from the genetic flock set aside specifically to provide lymphomatous material for inoculation. None of the birds in either of these groups had been inoculated nor had they had any contact with other inoculated birds. The pathological entities noted in the flock supplying chicks have been essentially the same during the past 7 years (5, 40). The incidence of visceral lymphomatosis has been about 25 per cent, the neural form about 10 per cent and ocular lymphomatosis less than 1 per cent. Erythrogranuloblastosis and osteopetrosis have been extremely rare.

For the series of inoculations reported herein, only cases that upon gross examination appeared to have lesions typical of visceral lymphomatosis were used. Microscopically, a diagnosis of visceral lymphomatosis was confirmed in all cases except F259F2. This bird had an enlarged liver and kidneys indicative of a diffuse lymphomatous involvement. However, microscopic examination revealed features not typical of those usually found in visceral lymphomatosis. The perivascular lymphocytic accumulations observed in the liver may have resulted from a reaction to bacterial infection. The glomeruli of the kidney were hypertrophied and no evidence of lymphomatosis was found.

Enlargement of peripheral nerves typical of neurolymphomatosis occurred in 4 donors (G124E, F615S2, F355D2, F404E). Microscopic study revealed 3 other birds with similar lesions (G736U2, G159K2, and F1110R). One donor, F1110R, also had ocular lymphomatosis.

The donors were chosen because of massive tumefaction of the liver or ovary. All had a lymphomatous liver and spleen and all but one had similar involvement of the kidney and ovary. Other tissues grossly or microscopically involved were, in decreasing order of frequency: bone marrow, pancreas, thymus, sciatic nerve, brachial plexus, adrenal gland, heart, intestine, skin, proventriculus, bursa of Fabricius, mesentery, muscle, pericardium, and eye.

In one bird (F1104G) the liver was diffusely tumorous. All others showed either a miliary type of involvement or focal tumors of varying size distributed throughout the liver parenchyma. Some livers were so tumorous that no parenchyma was grossly visible between the tumor areas. All ovarian tumors were classified as diffuse, since they were consistent lymphomatous masses with very little stroma.

Livers that were diffusely involved usually had a smooth surface and were pulpy or friable. Those with a focal type of involvement had a granular or irregular surface and were firm and resistant to section.

Microscopic study showed that the tumors were composed mainly of lymphocytes and larger immature cells which appeared to be hemocytoblasts (25). The proportion of hemocytoblasts making up the tumor varied considerably in the various donors. In F404E and F1104G, less than 1/10 of the cells making up the tumor could be classified as hemocytoblasts. In 5 others (G124E, G159K2, F355D2, F1110W, F1110R) 1/5 to 1/3 of the tumor cells were hemocytoblasts and in the remaining 3 cases (F615S2, F73602, F736U2) the proportion was about 2/3. The location of the tumor cells in the liver was almost entirely extravascular and extrasinusoidal except in one case. The involvement in the liver of G124E differed somewhat, in that part of the tumor was intrasinusoidal. One liver (G73602) had abundant fibrocytes and fibers within the tumor.

Examination of blood smears taken just prior to the collection of the tumors showed abnormal variations in all donors, but none could be classified as leukemic. A few donors had erythrocytes of abnormal size and shape with an appearance of polychrome erythrocytes and erythroblasts. The majority of donors had a slight to marked increase in the number of granulocytes. Myelocytes and hemocytoblasts were seen in a few of these cases; lymphocytes were decreased and abnormal thrombocytes appeared in most instances.

Inoculum.—All donors were killed by electrocution; tumor tissue for inoculation was removed with aseptic precautions. A cellular and a cell-free preparation were made from each tumor to be tested. The inocula containing viable cells were prepared by macerating the tumors (F615S2, F259F2, F355D2) in a mortar or in a mincer (34) and then suspending the mince in 4 parts of 0.85 per cent NaCl solution and filtering the suspension through a layer of sterile cheesecloth.

The cell-free inocula of tumors from donors F615S2, F259F2, F355D2, and G124E were prepared by homogenizing in a Waring Blendor[1] for 20 minutes with 9 parts of 0.85 per cent NaCl solution. The suspension was then spun in an angle centrifuge for 5 minutes at 1,700 RPM. The top 2/3 of the supernatant was slowly siphoned into clean tubes and again spun for 5 minutes at 1,700 RPM. The upper 2/3 of the second supernatant was used for inoculation. The cell-free inoculum of F404E was prepared in the same manner except that each centrifugation was made for 15 minutes at a speed of 3,000 RPM. The supernatant obtained from the second centrifugation at 3,000 RPM, was spun in an angle centrifuge for 3 hours at 19,000 RPM (a force of 27,000 times gravity). The resulting sediment that formed small pellets at the bottom of the tubes was resuspended in 1/8 the original volume of supernatant and used for inoculation.

The cell-free inocula of the remaining tumors were prepared by homogenizing the tissue with 9 parts of 0.78 per cent NaCl solution containing 0.03 M PO_4 buffer at pH 7.4 The suspension was then spun for 20 minutes in an angle centrifuge at 2,600-4,000 RPM. The supernatant was siphoned

[1] Obtained from the Central Scientific Co., Chicago, Ill.

off and passed through a preliminary (3 to 7 pounds pressure) Mandler filter. The cell-free filtrate of F1110R was, in addition, filtered through an 11 pound Mandler candle. This candle, after cleaning and resterilization, retained completely a 48 hour culture of *Serratia marcescens* filtered under the same conditions as the tumor extract. Precautions were taken to keep the tumor material within the temperature range of about 2° to 15° C. while the inoculum was being prepared and until it was injected into recipients.

All inoculations were made into the peritoneal cavity of chicks 1 day old. The dosage was 0.5 cc. for the cell suspension and 1.0 cc. for the cell-free inoculum.

Recipients.—Chicks for inoculation were obtained from pedigreed White Leghorn chickens maintained in quarantine and used in the genetic study of the disease (38). All chicks used in this study were obtained from those matings of line 15 (a line classified as relatively susceptible to lymphomatosis) that were relatively free from disease (39). Chicks injected with different inocula of the same donor were not kept separate; however, chicks inoculated with material from different donors were maintained in separate brooders, batteries, and pens.

Two groups of control chicks, obtained from the same matings and during the same period as the inoculated chicks, were maintained for the first 90 days in a quarantined pen with other chicks of the same origin which had not been inoculated or otherwise exposed to disease agents. During the age period of 90 to 183 days the controls were maintained with inoculated chicks of the same age. At 183 days of age all control birds were killed and examined for tumors in the same manner as the inoculated birds.

RESULTS

Preparations from 7 different lymphomatous livers, 1 spleen, 5 ovarian tumors, and the blood of 1 donor were tested. The results obtained for an experimental period of 183 days, except when otherwise indicated, are presented in Table I.

With certain exceptions, the pathological alterations obtained in the recipients were generally similar to those of the donors. Most of the tumors were confined to the viscera. One donor (F615F2) produced, in addition to the visceral tumors, a high incidence of osteopetrosis similar to that obtained with the lymphoid tumor strain RPL 12 (6). Only one case of osteopetrosis developed in the chicks injected with the cell-free preparation of the tumorous liver of G124E. Neither of these original donors showed any evidence of osteopetrosis. Several donors produced a few cases of neurolymphomatosis.

Of all the visceral positive cases, 95 per cent of the cellular inoculated and 98 per cent of the cell-free inoculated birds had lymphomatous livers. The spleen was tumorous in 83 per cent of cases in both groups and the kidney in 71 per cent in the cellular and 50 per cent in the cell-free inoculated group. The heart was positive in about 25 per cent and the gonad in about 10 per cent of all visceral positive cases in both the cellular and cell-free inoculated groups. Other organs were also occasionally affected. Occasionally the liver was diffusely involved with tumor tissue but generally the focal tumor areas of various sizes were distributed throughout the liver parenchyma.

The variation in transmission obtained with material from different donors was obvious. A significant incidence (63 to 85 per cent) of tumor formation was obtained with 7 of 14 cellular inocula and another inoculum produced tumors in 4 of 17 chicks. The incidence (14 and 21 per cent) of 2 other groups (ovary and liver of G124E) would very probably have been higher had they been kept for the full period (183 days) instead of only 93 days.

Only 5 of 13 cell-free preparations produced tumors in 39 to 94 per cent of the chicks. All tumors active in the cell-free form were also active as cellular preparations. Thus, there were preparations with three levels of activity: tumors from G124E, G73602, F615S2 and F1110R reproduced tumors with cellular and cell-free preparations; tumors from G736U2, G159K2, F404E, F1110W and F1104G were active only as cellular preparations; and tumors from F259F2 and F355D2 produced no tumors (1 case of neurolymphomatosis occurred among chicks inoculated with F259F2) with either preparation.

There appeared to be no obvious relation between the transmissibility of the tumor and its location. Tumorous livers occurred in all 3 classes of activity and ovarian tumors appeared in both the cell-free and cellular categories.

A high incidence of enlarged bones typical of osteopetrosis was obtained with inocula of F615S2. This change was conspicuously absent from all other groups, with the exception of a single bird inoculated with the cell-free extract of G124E.

Enlargement of the nerves typical of neurolymphomatosis occurred in a few birds of several groups. The highest incidence of nerve involvement was obtained with heparinized whole blood and cell suspension of the ovarian tumor of F404E.

Each inoculum produced 4 cases of neural involvement in 20 birds inoculated, but involvement of the viscera was conspicuously absent. Two to three cases of neurolymphomatosis also occurred in 4 other cellular inoculated groups, but only one case developed in each of 3 of 13 injected with cell-free preparations.

duce similar tumors in recipients, thus indicating that there is a filtrable agent in tumors of visceral lymphomatosis which is capable of reproducing lymphoid tumors in chickens. It is significant that no evidence of tumor was found in chickens of the 2 control groups. This result, and the fact that no pathological finding was obtained among the birds

TABLE I: RESULTS OF INJECTING YOUNG CHICKS WITH CELLULAR AND CELL-FREE INOCULA PREPARED FROM TUMORS OF VISCERAL LYMPHOMATOSIS

| Donor | | Inoculum | | Number inoculated | Number with tumors of the | | | Total Pos. cases | | Age at death of visceral positive cases, days | |
Bird number	Age, days	Source	Type		Bone	Viscera	Nerve	Number	Per cent	1st case	Average
G124E	113	Ovary,	cellular	14*	0	2	0	2	14	70	75
			cell-free	15	0	10	1	11	73	74	117
		Liver,	cellular	14*	0	3	0	3	21	50	77
			cell-free	17	1	12	0	12	71	76	128
G736U2	176	Liver,	cellular	18	0	11	2	13	72	33	97
			cell-free	20	0	0	0	0	0	..	..
G73602	197	Liver,	cellular	19	0	12	3	13	68	26	133
		Spleen	"	19	0	14	0	14	74	22	51
		Kidney) Liver }Spleen)	cell-free	18	0	8	0	8	49	66	116
G159K2	260	Liver,	cellular	13	0	11	0	11	85	108	149
			cell-free	18	0	0	0	0	0	..	..
F615S2	392	Ovary,	cellular	14*	7	5	0	11	79	63	70
			cell-free	16	10	12	1	15	94	79	123
F355D2	477	Liver,	cellular	20*	0	0	0	0	0	..	..
			cell-free	21	0	0	0	0	0	..	..
F259F2	532	Liver,	cellular	21*	0	0	1	1	5	..	..
			cell-free	20	0	0	0	0	0	..	..
F404E	574	Blood,	cellular	20	0	0	4	4	20	..	..
		Ovary,	"	20	0	0	4	4	20	..	..
			cell-free	19	0	1	0	1	5	..	..
			centri. sed.	19	0	0	0	0	0	..	..
F1110W	742	Ovary,	cellular	19	0	11	2	12	63	32	140
			cell-free	17	0	0	0	0	0	..	..
F1104G	811	Ovary,	cellular	17	0	4	0	4	24	14	126
			cell-free	19	0	0	0	0	0	..	..
F1110R	812	Liver,	cellular	18	0	12	2	14	78	78	135
			cell-free	18	0	6	1	7	39	137	145
Controls (Contact after 90 days)				20	0	0	0	0	0	..	..
" " " " "				21	0	0	0	0	0	..	..

* Experimental period of 93 days, all others 183 days.

DISCUSSION

The results presented show that cellular inocula prepared from the visceral tumors of certain cases of naturally occurring visceral lymphomatosis produced similar tumors in inoculated chickens. These results are similar to those reported earlier by Burmester and Prickett (5) and supplement them. Together with the results of Brewer and Brownstein (4) they show beyond doubt that many cases of naturally occurring lymphomatosis are readily transplanted to healthy chicks, and indicate that the tumors of Olson (35) and Pentimalli (36) may not be rare types of transplantable lymphoid tumor.

Data presented herein also show that cell-free inocula (in 2 instances a filtrate) prepared from tumors of certain cases of visceral lymphomatosis will in-

of 7 groups comprising 134 individuals which had been inoculated with cell-free material, would indicate that the positive cases obtained were entirely to the inoculation and not due in part to possible prior infection of the chicks or to environmental factors.

Furth (21) has reported that a virus produced an unusual type of lymphomatosis in chickens, and Burmester, Prickett, and Belding (6) have shown that filtrates from a lymphoid tumor derived from a case of lymphocytoma (35) produced a high incidence of osteopetrosis and lymphomatous tumors of the viscera. However, a survey of the literature indicates that this is the first report showing that cell-free inocula prepared from several cases of naturally occurring visceral lymphomatosis pro-

duced a highly significant incidence of similar tumors in a relatively short time.

Since similar results were obtained when the inoculum was passed through a bacteria-retaining Mandler filter (F1110R) it may be suggested that the tumors found in cases of naturally occurring lymphomatosis may be transmitted by a filterable agent.

The variation in the transmission data between donors and types of preparation was a characteristic feature of these results. While 14 of 7 donors (No. 2 gave negative results with both the cellular and cell-free preparations. One of these, J 264 must be omitted from the consideration since it was found to be atypical of lymphomatosis. The cellular preparation from at the donors 14 of 15 produced a 20 per cent incidence of visceral tumors. The cellular preparation of 5 of these donors produced a significant incidence of visceral tumors, but cell-free preparations from only 4 of these were active. Since an active filterable agent was evidenced in preparations from 4 donors that produced a cellular preparation was as suggested that a filterable agent played little if any part in the formation of tumors when cellular stocks were used. However, the lack of transmission with a filtrate is not in itself proof that some form of agent was not present. Evidence for this was obtained in the case of G754K. The cellular material of G754 taken from the liver of this donor produced 14 visceral tumors among 18 inoculated chicks and the cell-free preparation failed to produce any tumors. However, a filtrate prepared from the fifth cellular passage of this tumor induced a high incidence of visceral lymphoid tumors and osteopetrosis (13, 8) indicating that an agent or agents must have been present in either passage in a decreased or an inactive form. Similar phenomena have been observed by others (8, 9, 11) with the Rous tumor virus and suggested for other viruses (7). Variations in the type of reactions obtained are also found in the age at death of the first animal with a visceral tumor for each inoculum. The earliest case occurred in 14 days after inoculation with the cellular preparation of F1104G. However the total incidence during a 154 day period was only 33 per cent. In contrast, G650K2 was the slowest (115 days) in producing tumors with cellular material but the incidence of tumors of the viscera was highest 85 per cent) by the time the birds were 154 days of age. Thus there appeared to be a factor or factors influencing the speed of tumor development, which may have been independent of factors that determined the number or percentage of tumor

transplantability of tumors. He concluded that transplantation and active filtrates were more often obtained with tumors from chickens that were 5 to 10 months of age than from chickens 1 to 4 or 12 to 24 months of age. These results were based entirely on sarcomas since he was unsuccessful in obtaining any transplantation of carcinomas and lymphoid tumors (11).

If the data reported earlier (5) are combined with those presented herein and arranged in order of ascending age, it will be found that tumors from the 8 donors 72 to 392 days of age, and those from 3 donors 742 to 812 days of age, were all transplantable; whereas only 1 of 5 tumors from donors 407 to 574 days of age were transplantable. The transplantability of some of these tumors may be open to question since only one passage was made for part of them (for the remaining, several passages were made [5, 7]) and the incidence of tumors was low in a few inoculations.

Although the low number of donors in the oldest age group precludes an absolutely reliable prediction of results in this group, the data do suggest that tumors of birds of a certain age range (407 to 514 days) are less likely to be transplantable than tumors of birds outside this range. These results are not in agreement with the general idea of Duran-Reynals (10) that tumors are less likely to be transplantable when they are from young or old hosts; however, his age ranges were much narrower, since his medium (or active) range (5 to 10 months) was entirely within the low (also active) age range (72 to 386 days) presented herein. Furthermore, a significant difference in Duran-Reynals' low age group (Group III) and the medium age group (Group II) is not apparent. Although only 1 of the 5 tumors was transplanted for 2 passages, 3 of them produced tumors in the first passage and a critical test was not obtained because the number of chicks used in most cases was small and in 2 instances several chicks died "prematurely" or "accidentally." More extensive data must be accumulated before conclusions can be drawn with regard to the relation between the transplantability of naturally occurring chicken tumors and age of the tumor-bearing animal.

Donors used in this study were chosen because of the presence of lymphomatous tumors in the viscera; however, 7 of the 10 donors also showed gross or microscopic lymphoid accumulations in the peripheral nerves. Three of the 7 donors having neurolymphomatosis produced 2 or more cases with nerve involvement, 2 donors produced 1 case each, and the remaining 2 failed to produce any. Of the 3 donors with no gross or microscopic evidence of

neurolymphomatosis, the recipients of 2 of these had two or more cases of neurolymphomatosis and the recipients of the remaining donor failed to develop any cases (Table I). Thus, there is no indication of a relation between the presence of lymphoid accumulations in the nerves of donors and the presence or incidence of similar alterations in recipients, though there is some indication in these data that the agent of visceral lymphomatosis is different from that of neurolymphomatosis. A comparison of the effectiveness of cellular and cell-free inocula in producing tumors reveals that cellular inocula produced an incidence of visceral tumors 1.7 times greater than cell-free preparations, whereas the same cellular inocula produced an incidence of neurolymphomatosis 6.0 times greater than the cell-free preparations. Further evidence is found in the results obtained with the preparation of F404E. The blood of this donor and the cell suspension of the ovarian tumor produced 4 cases of neurolymphomatosis in each of 2 groups of 20 chicks injected, whereas no cases with visceral tumors developed, thus indicating that a neurolymphomatosis-inducing agent was present but the visceral tumor agent was either absent or present in a masked or inactive form. Separate etiology for the two manifestations has been suggested or implied by several investigators (12, 19, 22), though conclusive evidence is still lacking.

SUMMARY AND CONCLUSIONS

1. The transmissibility of tumors from 10 cases of naturally occurring visceral lymphomatosis was tested by inoculation of cellular and cell-free preparations into groups of 13 to 21 day-old chicks.

2. Lymphomatous tumors of the viscera were reproduced by cell-containing preparations from 8 of the original tumors in 14 to 85 per cent of chicks in 93 to 183 days. Similar tumors were produced by cell-free preparations from 4 of the original tumors in 39 to 94 per cent of the chicks in 183 days. None of 41 noninoculated controls developed lymphomatosis during the same experimental period. Thus, tumors of some, but not all, cases of visceral lymphomatosis are transplantable, and part of these tumors may be transmitted to chicks by inoculation with filtrates. The active agent or agents appear to be of a size which will allow them to pass readily through bacteria-retaining filters.

3. Of the 10 donors that supplied visceral tumors, 7 also had gross or microscopic evidence of neurolymphomatosis. There appeared to be no direct relation between the presence of this lesion in the donor and the number of recipients that developed neural or visceral lymphomatosis.

Studies on the Transmission of Avian Visceral Lymphomatosis
II. Propagation of Lymphomatosis with Cellular and Cell-Free Preparations

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INTRODUCTION

Research on lymphomatosis in chickens has been greatly retarded by difficulties experienced in experimentally reproducing the disease at a relatively high rate. Furth (12, 13), Pentimalli (24), and Olson (22) propagated lymphoid tumors by intramuscular transplantation. Although the tumors were derived from naturally occurring cases indistinguishable from lymphomatosis, they were considered by the authors as rare or atypical because of their transmission characteristics. More recently, Burmester and Prickett (2) obtained 4 lymphoid tumor strains from 9 different inocula prepared from 6 original donors, thus indicating that the procurement of rapidly growing tumors from naturally occurring cases of visceral lymphomatosis was not difficult. This result was confirmed by Brewer and Brownstein (1).

Although Lucas (19) has shown a possible means of cell transmission from parent to offspring, he indicated that there were no data to support this idea. It is doubtful that the disease in nature is due to transplants. However, it was shown by Burmester, Prickett, and Belding (3) that the rapidly growing tumor developed by Olson (22) contains a filtrable agent or agents which produce osteopetrosis and lymphoid tumors of the viscera. If similar agents are found in other rapidly growing lymphoid tumor strains, then tumor strains become a valuable source of filtrable agents directly associated with visceral lymphomatosis.

The purpose of this report is to show that filtrates prepared from tumors of several strains recently developed from cases of naturally occurring visceral lymphomatosis, and propagated with transplants or with filtrates, produced a high incidence of visceral lymphomas indistinguishable from similar tumors in cases of visceral lymphomatosis. The characteristics of 4 new lymphoid tumor strains obtained from cases of visceral lymphomatosis are also presented.

PROPAGATION OF TUMOR STRAINS RPL 18, 19, 20 AND 21 WITH CELLULAR INOCULUM

Materials and methods.—Donors for the propagation of the tumor strains were selected from groups of birds inoculated with the same strain or with material from a naturally occurring case of lymphomatosis described by Burmester and Denington (5). Attempts were made to obtain cases which appeared early. Lymphomatous liver tissue was used except when otherwise indicated. The tissue was pressed through a fine screen and suspended in 3 parts of 0.85 per cent NaCl solution. The suspension was filtered through cheesecloth and injected with a syringe and needle into the peritoneal cavity of chicks in doses of 0.1 to 0.5 ml.

Chicks for inoculation were obtained from matings of pedigreed White Leghorn chickens maintained in quarantine and used in the genetic study of this disease (25). With minor exceptions, they were obtained from those matings of line 15 (a line classified as partially susceptible to lymphomatosis) which were relatively free from disease (26).

Results.—Tumors originating from 4 different original donors (5) were propagated in serial passage with cellular material for 5 to 15 passages. The origin and type of inoculum and the relation between series for each strain are presented diagramatically in Fig. 1. A summary of the transmission results obtained with the cellular inocula in serial passage is given in Table I.

Series A of Strain RPL 18 arose with the cellular inoculum of the original ovarian tumor of F615S2, and series B came from the cell-free preparation of the same tumor (Fig. 1). The transmission characteristics of the two series were very similar. Bone and visceral tumors occurred in the first passage of both series, after which tumors were confined primarily to the liver, spleen, and kidney. The tumor incidence was high in all inoculations of both series, and the passage interval decreased from 79 days to 7 to 12 days (series B) and 6 days (series A). A similar reduction in the average survival period also took place.

Strain RPL 19 originated with the cell-free preparation of the ovarian tumor of G124E. After the first passage for series A and the second passage for series C, all inoculations were made with cellular suspensions (Fig. 1). Tumors in series A were confined to a lymphoid involvement of several

Passages   1   2   3   4   5   6   7   8
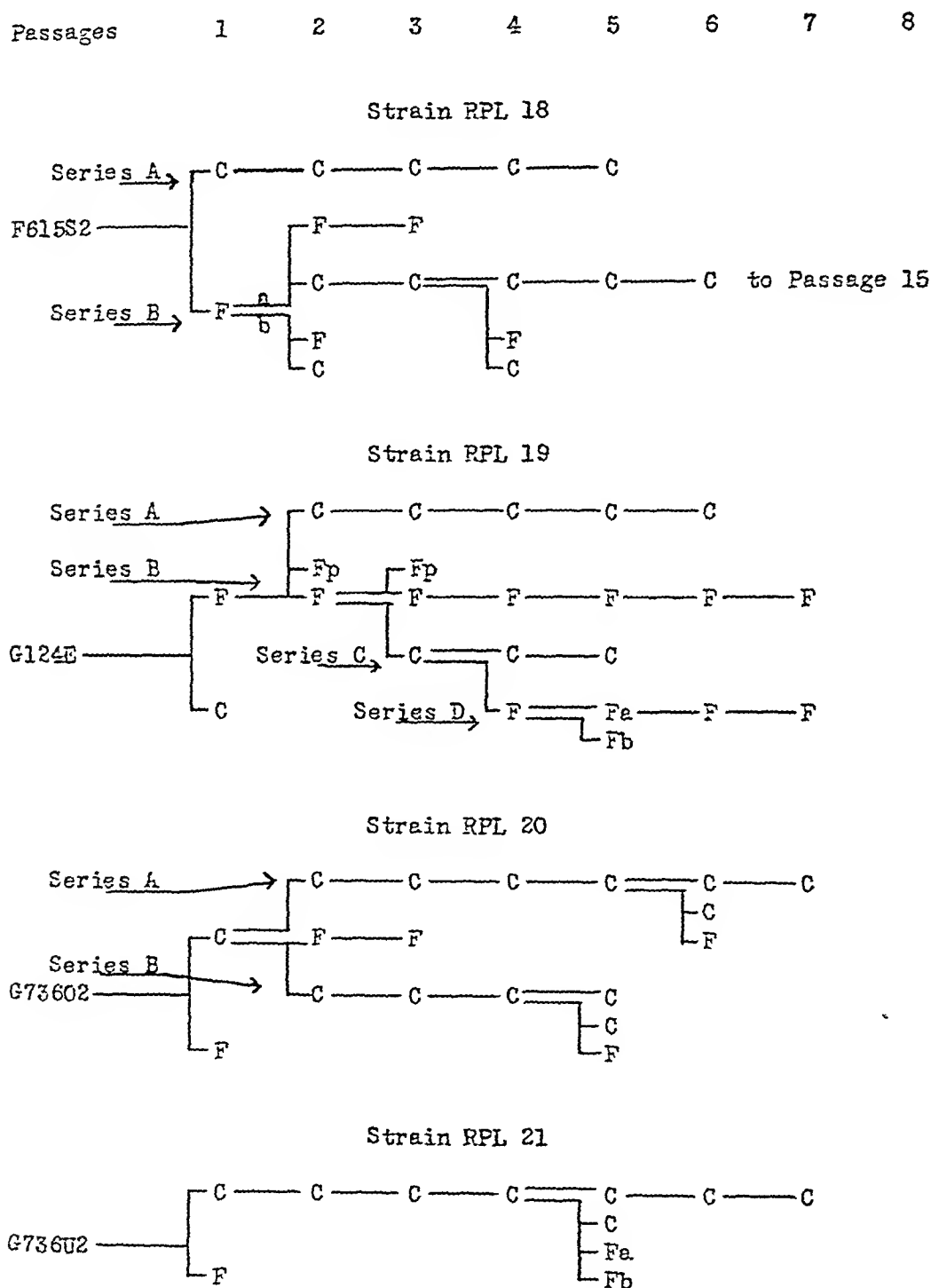
Fig. 1.—Diagrammatic presentation of inocula and passages of Strains RPL 18, 19, 20, and 21. C = Cellular, F = Cell-free, and b = 2 preparations. p = plasma.

visceral organs. In series C, in addition to the lymphoid involvement, hemangiomas 1 to 2 cm. in diameter were found in 3 cases of the third passage. The selection of donors without angiomas resulted in absence of this characteristic in the fourth and fifth passages. The incidence of tumors was very high in all passages except the fifth and the sixth of series A. The reason for the low incidence in these passages may be the fact that chicks of line 15 were not available at the time these passages were made, and birds from several other lines (lines 7, 9, 11) were used. A greater inherited resistance in the latter chicks, or the fact that heterologous transplants were made, may account for the low incidence; however, it did not appear to affect the rate of tumor development. Birds that developed tumors died in a short time (average 7 to 11 days) and transfers were made in 7 to 9 days. Although a high incidence of tumors was obtained in passages 3, 4, and 5, of Series C, the tumors developed at a

TABLE I: SUMMARY OF DATA OBTAINED IN SERIAL PASSAGE OF STRAINS RPL 18, 19, 20, AND 21 WITH CELLULAR INOCULA

Passage number	Age of recipients at inoculation, days	Number chicks inoculated	No. with tumors of the Bone	Viscera	Nerve	Total no. of positive cases	Av survival of visc. cases, days	Passage interval, days
STRAIN RPL 18 (FROM OVARIAN TUMOR OF F615s2)								
Series A†								
1–C*	1	14	7	5	0	11	70	79
2–C	2	18	0	12	2	14	135	146
3–C	8	9	0	6	2	8	37	14
4–C	22	5	0	5	0	5	7	7
5–C	15	5	0	5	0	5	5	6
Series B								
1F*	1	16	10	12	1	15	123	79
2–C	10	9	0	8	0	8	79	37
3–C	12	14	0	11	0	11	24	10
4–C	22	14	0	13	0	13	14	8
5–C	30	13	0	10	0	10	30	12
6–10–C	28–60	39	0	35	0	35	14	7–11
11–15–C	38–75	25	0	20	0	20	11	8–12
STRAIN RPL 19 (FROM OVARIAN TUMOR OF G124E)								
Series A								
1–F*	1	15	0	10	1	11	117	81
2–C	4	9	0	8	0	8	19	12
3–C	2	9	0	9	0	9	8	7
4–C	9	8	0	5	0	5	11	9
5–C	18	5	0	2	0	2	7	7
6–C	25	5	0	1	0	1	9	..
Series C								
3–C (liver)	15	10	0	9‡	0	9	30	..
3–C (blood)	15	10	0	10	0	10	16	12
4–C	27	10	0	10	0	10	16	12
5–C	39	10	0	7	0	7	20	..
STRAIN RPL 20 (FROM LYMPHOMATOUS SPLEEN OF G73602)								
Series A								
1–C*	1	19	0	14	0	14	51	22
2–C	8	20	0	14	0	14	51	22
3–C	16	15	0	5	0	5	69	16
4–C	4	12	0	5	0	5	33	11
5–C	15	10	0	5	0	5	22	14
6–8–C	15–24	30	0	14	0	14	22	15
Series B								
2–C	1	9	0	8	0	8	21	16
3–C	4	13	0	11	0	11	22	11
4–C	15	10	0	7	0	7	27	14
5–C	15	10	0	6	0	6	27	..
STRAIN RPL 21 (FROM LYMPHOMATOUS LIVER OF G736U2)								
1–C*	1	18	0	11	2	13	97	33
2–C	5	9	0	4	0	4	31	19
3–C	4	13	0	13	0	13	17	11
4–C	15	10	0	9	0	9	17	14
5–C	15	10	0	7	0	7	15	14
6–8–C	22–35	29	0	22	0	22	15	7–13

* Results of the first passage were presented previously (3).
C = Cellular inoculum, F = Cell-free inoculum.
† *See* Fig. 1 for relationship of various series and inocula used for various passages.
‡ Three cases developed hemangiomatosis also.

slower rate, giving an average age at death of 16 to 30 days for the 3 transfers.

Strain RPL 20 originated with the lymphomatous spleen of G736O2. All subsequent passages were made with liver tissue from inoculated birds. Series B began with a different donor from series A in chicks of the first passage, and was maintained separately for four transfers (Fig. 1). Characteristics of the two series were essentially the same. Tumors were confined to the viscera, but were somewhat different from tumors of other strains in that a considerable amount of fibrosis was present in lymphomatous livers. (See section on pathology.) The incidence of tumor involvement in Strain 20 was not so great as in other strains, nor did it appear to cause the death of inoculated birds so rapidly. In series A the highest incidence (74 per cent) was in the first passage and an average of only 47 per cent for the sixth to eighth passages. The average survival period varied from a maximum of 69 days in the third transfer (series A) to 21 days in the second passage (series B). Within series A the survival period was much shorter in the later passages than in the first three. The comparatively long survival period for birds implanted with Strain RPL 20 may in part account for the greater amount of fibrosis found in livers of this avian strain.

Strain RPL 21 originated with the lymphomatous liver of G736U2. Except for 2 cases of neurolymphomatosis, which occurred in the first passage, the involvement in all other positive birds was confined to the viscera. The percentage of birds that developed tumors ranged from a low of 45 per cent for chickens of the second passage to 100 per cent for the third passage, a mean of 76 per cent for passages 6, 7, and 8. There was a consistent decrease in the average survival period of from 97 days for the first passage to 15 days for the fifth to eighth passages.

THE PROPAGATION OF STRAIN RPL 19 WITH FILTRATES AND CELL-FREE PREPARATIONS

Materials and methods.—Donors used for the preparation of cell-free material were chosen on the same basis as those used for serial transfers with cellular inoculum. Tumorous liver tissue was used in the preparation of inocula for serial passage. Plasma from birds with tumorous livers was employed in supplementary inoculations of passages 2 and 3.

The preparation of the inoculum used for the first passage (centrifuged extract of the ovarian tumor of G124E) has already been described (5). Tumor tissue for cell-free inocula used in passages 2 and 3, was homogenized with 7 parts of 0.85 per cent NaCl solution in a Waring Blendor for 12 minutes. The suspension was then spun in an angle centrifuge at 2,600 RPM. The upper two-thirds of the supernatant was siphoned into clean tubes and recentrifuged. The upper two-thirds of the second supernatant was carefully removed and used as the inoculum. Plasma used in the same passages was obtained from blood of tumor-bearing birds; it was separated by a centrifugation procedure similar to that used for the liver suspension.

Inocula for other passages in series B, and all passages of series D (Fig. 1). except 6. were prepared in the following manner. The tumorous liver tissue was homogenized 8 to 12 minutes with 7 parts of 0.78 per cent NaCl solution containing 0.03 M PO$_4$ buffer at pH 7.4. The suspension was spun in an angle centrifuge at 4,500 RPM. The supernatant was then filtered through a regular porosity (6 to 9 lbs. air pressure) Mandler candle. Filters used for passages 6 and 7, series B; and 4, 5, and 6 of series D; were tested for their retention of *Serratia marcescens*. All tests were made with a heavy suspension of the organism in broth and under filtration conditions similar to those used for the inocula. They were made either immediately following the filtration of the inoculum or after the filters had been re-cleaned and sterilized. Cultures of the filtrates and the filtrates themselves were sterile after 72 hours incubation; whereas heavy growth was obtained in cultures of unfiltered broth suspensions.

The inoculum used for passage 6, series D, was prepared in the manner described except that it was not passed through a filter. Inoculations were made by the intraperitoneal route with 0.5 to 1.0 ml. into chicks of line 15 described in a previous section.

Results.—Transmission results obtained in the serial passage of Strain RPL 19 with cell-free material in one complete series, and one partial series, are summarized in Table II. In series B, after the first passage with cell-free material, a high percentage of birds with lymphoid involvement of the liver also had innumerable hemangiomas located in the loose connective tissue about the skeletal muscle in the subcutis and serosal membranes. Hemangiomatosis appeared in chicks of all inoculations and passages of series B, except for the first passage, and those injected with plasma in the second passage. There were only 2 cases (sixth passage) of angiomas, which did not show gross evidence of lymphoid involvement, and several in all passages which had lymphoid involvement without gross evidence of hemangiomatosis. The average incidence

of death from tumors for passages 4 to 7 inclusive, series A, was 92 per cent. In the same passages, the average survival period varied from 23 to 46 days with a passage interval of 15, 20, and 34 days.

The purpose of series D was to establish a substrain without hemangiomatosis. Chicks for passage 4, series D, were inoculated with the filtrate of a hemangioma-free donor (passage 3, series C) which had previously been inoculated with a cell-suspension of a bird of the second passage, series B (Fig. 1). The latter donor also had no gross

lations. The plasma used in the second and fourth passage test was collected from birds with gross involvement of the viscera by withdrawing blood from the heart with a syringe containing 0.4 per cent solution of heparin, equivalent to one-tenth the final volume of blood withdrawn. The blood cells were separated by centrifugation and the plasma filtered through a Seitz S1 filter pad for passage 2, and a Fisher-Jenkins filter for passage 4.

For the preparation of filtrates, tumorous livers were homogenized in a Waring Blendor with 9

TABLE II: SUMMARY OF DATA OBTAINED IN THE SERIAL PASSAGE OF STRAIN RPL 19 WITH CELL-FREE INOCULA

Passage Number	Inoculum	Age of recipients at inoculation, days	No. chicks inoculated	No. with tumors of the Nerve	No. with tumors of the Viscera	No. with hemangiomas	Total no. pos. cases	Av. survival of pos. cases, days	Passage interval, days
Series B									
1	Ovary — H,C*	1	15	1	10	0	11	117	81
2	Liver — H,C	4	13	0	6	2	6	62	56
	Plasma — C	4	16	0	2	0	2	86	..
3	Liver — H,C	4	10	0	10	7	10	54	32
	Plasma — C	4	10	0	8	2	8	59	..
4	Liver — H,C,F	1	17	0	17	15	17	23	20
5	Liver — H,C,F	7	18	0	16	14	16	38	15
6	Liver — H,C,F	22	19	0	15	12	17	46	34
7	Liver — H,C,F	21	19	0	17	11	17	43	..
Series D									
4	Liver — H,C,F	1	13	0	12	2	12	39	12
5a	Liver — H,C,F	4	10	0	8	0	8	28	14
5b	Liver — H,C,F	4	12	0	11	0	11	40	..
6	Liver — H,C	4	16	0	14	0	14	20	11
7	Liver — H,C,F	8	14	0	14	0	14	21	13

* H = Homogenized,　C = Centrifuged at 1,700–4,000 RPM,　F = Filtered.

evidence of hemangiomatosis though the inoculum from this donor produced 3 cases (Table I) among 10 birds inoculated.

Among the chicks of the fourth passage, only 2 developed hemangiomas; whereas 12 of 13 inoculated developed lymphoid tumor and died at the average age of 39 days. Hemangiomatosis did not appear in the succeeding 3 passages, but with a high incidence of lymphoid tumor development (80 to 100 per cent), short average survival period (20 to 40 days), and rapid passage (11 to 14 days), remained characteristic of this strain.

ACTIVITY OF CELL-FREE FILTRATES OF STRAINS RPL 18, 20, AND 21.

Materials and methods.—Birds used to supply material for the filtrability tests were typical of those that showed advanced lesions of the respective strains, with the exception of one donor, H1308X, which was used in the third passage test of Strain RPL 20 and, in contrast to all other donors, showed no gross tumor involvement.

Plasma and lymphomatous liver tissue were used as source material for Strain RPL 18 filtrate inocu-

parts of buffered saline. The suspension was spun in an angle centrifuge and the supernatant passed through a preliminary Mandler candle in passages 2 and 3, and a regular candle in passage 4.

Strain RPL 20 filtrates were prepared in a similar manner. A regular Mandler candle was used in passage 3 and the fine grade was used in passages 2, 4, and 5. The centrifuged sample tested in passage 5 was prepared by recentrifuging the first supernatant for 15 minutes at 4,500 RPM. The resulting supernatant was then carefully siphoned into a bottle and inoculated into chicks.

The cell-free inoculum used in Strain RPL 21 filtrability test was prepared in the same manner as the RPL 20 filtrate, using a regular Mandler filter. The high speed sedimented inoculum was obtained after further centrifugal fractionation. The supernatant obtained after centrifuging the tumor suspension was transferred to Lusteroid tubes and spun in an angle centrifuge for 2½ hours at 19,000 RPM (R.C.F.=27,000 g). The supernatant was discarded and the small sedimented pellet was resuspended with buffered saline and diluted to ¼ the original volume. The undissolved particles

were resedimented by spinning for 10 minutes at 19,000 RPM. The supernatant was then used for inoculation of chicks.

All Mandler filters, with the exception of the ones used for RPL 18, passage 3; and RPL 20, passage 2 were tested for their ability to retain *Serratia marcescens*. Only the preliminary candle used for

lum for passage 3, Strain RPL 18 was prepared and chicks of lines 7, 11, and 13 (24) were used.

All inoculations were made into the peritoneal cavity in doses of 1.0 ml. for the filtrates and 0.5 ml. for the cellular preparations. The birds were maintained for a period of 186 to 200 days.

Results.—Tumors that developed in the chickens

TABLE III. RESULTS OF INOCULATING CHICKS WITH CELL-FREE FILTRATES AND CELL-SUSPENSIONS OF TUMORS OBTAINED IN SERIAL PASSAGE OF STRAINS RPL 18, 20, AND 21

Passage no	Inoculum	No. chicks inoculated	No. with tumor of			Total no. pos. cases		Av. survival of visc. cases, days
			Bone	Viscera	Nerve	No.	%	
	STRAIN RPL 18							
	Cell-free							
1*	Liver — H,C†	16	10	12	1	15	94	123
2a	Plasma — H,C,F	18	0	10	0	10	56	125
2b	Liver — H,C,F	19	1	15	0	15	79	128
3	Liver — H,C,F	14	0	2	1	2	14	139
4	Liver — H,C,F	12	1	9	0	9	75	148
4	Plasma — H,C,F	6	0	5	0	5	83	128
	Cellular							
2a	Whole blood	9	2	4	0	5	56	132
2b	Liver cell susp.	9	0	8	0	8	89	79
4	Liver cell susp.	14	0	13	0	13	93	14
	STRAIN RPL 20							
	Cell-free							
1*	Pooled — H,C	18	0	8	0	8	49	116
2	Liver — H,C,F	7	0	0	0	0	0	..
3	Liver — H,C,F	12	0	8	0	8	67	141
5	Liver — H,C,F	15	0	14	0	14	93	154
6	Liver — H,C	16	0	8	0	8	50	148
	Liver — H,C,F	16	0	10	0	10	63	152
	Cellular							
2	Liver cell susp.	9	0	8	0	8	89	21
5	Liver cell susp.	5	0	5	0	5	100	18
6	Liver cell susp.	5	0	5	0	5	100	100
	STRAIN RPL 21							
	Cell-free							
1*	Liver — H,C	20	0	0	0	0	0	..
6	Liver — H,C,F	15	11	11	0	13	87	159
	H,C,P	13	10	11‡	4	13	100	162
	Cellular							
6	Liver cell susp.	8	0	8	0	8	100	15
	NON-INOCULATED CONTROLS							
	Group A None	17	0	0	0	0	0	..
	Group B None	17	0	0	0	0	0	..

* Results of the first passage presented previously (5).
† H = Homogenized, C = Centrifuged at 1,700–4,000 RPM, F = Filtered, P = Purified.
‡ One of these had ocular lymphomatosis also.

the second passage of RPL 18 was found to allow the passage of bacteria.

Cell suspensions of tumorous livers, used in some inoculations to give an indication of their transplantability, were prepared in like manner as similar preparations used in the serial passage of the several strains.

Day-old chicks from matings of line 15 (a lymphomatosis-susceptible line), which had a relatively low incidence of the naturally occurring disease, were used in all inoculations except one; chicks from this source were not available when the inocu-

were classified with respect to location. A few birds developed an enlargement of the peripheral nerves typical of neurolymphomatosis, others inoculated with filtrates of Strains 18 and 21 developed osteopetrosis. The majority of the birds that died had lymphoid tumors in the viscera. Transmission results obtained with the various preparations are presented in Table III.

The cell-free preparation of the tumor from the original donor of Strain RPL 18 (F615S2) produced a high incidence of tumors. Of 16 chicks inoculated, 10 developed osteopetrosis, and 12, tu-

mors of the viscera with an average survival period of 123 days. Two birds that were shown by gross examination to have osteopetrosis were the source of blood for passage 2a. The whole blood produced osteopetrosis and visceral tumors, whereas the filtered plasma produced only lymphoid tumors of the viscera. A bird of the first passage having only tumors of the viscera was used as the source of inoculum for passage 2b. The cell suspension produced a high incidence of visceral tumors without any indication of osteopetrosis, whereas the filtrate caused the appearance of bone involvement in one case and visceral tumors in 15 of 19 chicks inoculated. One of the latter, with massive involvement of the liver, spleen, and kidneys, was used as the donor for passage 3. This inoculum produced tumors in only 2 of 14 chicks inoculated; which low incidence may have been due to a higher natural resistance of the chicks, since sources other than line 15 were used.

Birds of the third cellular passage, series B, Strain RPL 18 (Fig. 1), were the source of material used in the inoculation designated as the fourth passage. A high incidence of visceral tumors developed among birds injected with filtered plasma, filtered liver extract, and with a cellular suspension of the liver. Chicks injected with the latter died in 14 days, whereas those that received filtered material died on an average of 128 and 148 days after inoculation. Difference in the age at death between chicks inoculated with cellular and cell-free material was much less in the passage 2b, and was absent in inoculation 2a.

The cell-free preparation of tumors of the original donor of Strain RPL20, G73602, produced visceral tumors in 8 of 18 birds inoculated (5). The second passage test was made with the lymphomatous liver of a chicken 43 days after its inoculation with a cell suspension of the original donor (Fig. 1). Of 7 birds inoculated, 2 died from unknown causes at 17 and 20 days of age, and one, G1308X, though showing no gross evidence of lymphoid involvement, was used at 21 days of age as a donor for the third passage. The remaining 5 lived until the termination of the experiment. The cell suspension prepared from the same liver produced death with tumors of the viscera in 8 of 9 chicks inoculated, in an average of 21 days.

The donor of the third filtrate passage of this strain, G1308X, though without gross or microscopic evidence of tumors, had a number of cells, indistinguishable from the typical tumor cell, in the sinusoids of the liver and in the myocardium. Filtered preparations of the liver produced typical lymphomatous tumors in the viscera in 8 of 12

birds inoculated and the tumorous birds died in an average of 141 days after inoculation.

The tumor used for the fifth filtrability test came from the fourth cellular passage, series B (Fig. 1). The Mandler-filtered preparation produced massive lymphoid involvement of the visceral organs in 14 of 15 birds inoculated, and death in 154 days; whereas cell suspension prepared from the same liver produced tumors and death of all birds in 18 days.

For the sixth passage test, tumorous liver from a case of the fifth cellular passage, series A, was used (Fig. (1). The centrifuged material produced 8, and filtered inoculum 10, cases with tumors of 16 injected with each preparation, with average age at death of 148 to 152 days, respectively. The cellular inoculum produced tumors in all 5 birds and death at an average of 109 days. The individual survival periods were 22, 26, 136, 165 and 198 days. The same cellular suspension injected into another group of 10 birds, 15 days of age, produced tumors in 6 and caused death in 14 to 27 days (average 21 days). Since cell-free preparations from the same tumor produced death in an average of 148 to 152 days, one may infer that the neoplastic cells injected into the peritoneal cavity of the 3 birds with a survival of 136, 165, and 198 days, did not grow into tumors, but the tumors found at death in these birds were due to the action of the filtrable agent present in the cellular inoculum.

The lymphomatous liver used as source material for the filtrability test of RPL 21 was obtained from a bird recently implanted with cell suspension of the fifth passage of this strain. The cell suspension produced tumors of the viscera in all birds and caused their death in 15 days (average); whereas osteopetrosis and/or visceral tumors developed in a high percentage (87 per cent) of the chicks injected with the filtrate. An extract of the high speed centrifuged pellet also produced a high incidence of osteopetrosis and visceral tumors, but, in addition, 4 of the 13 inoculated birds were diagnosed as having neurolymphomatosis and one of them had ocular lymphomatosis. The average age at death of the latter 2 groups (average of 159, 162 days) was similar to other groups injected with cell-free preparations of Strains RPL 18 and 20.

Two groups of chicks obtained from the same matings, and hatched at about the same time as the inoculated chicks, were maintained for the first 90 days in isolation with other non-inoculated chicks of the same source. During the period of 90 days of age to the time they were killed for necropsy, 200 days for Group A and 186 days for

Group B, they were maintained with inoculated birds. No evidence of tumors was found in any birds of either group.

THE PATHOLOGICAL CHARACTERISTICS OF TUMOR STRAINS RPL 18, 19, 20, AND 21

The main feature of all strains obtained from original donors having lesions typical of visceral lymphomatosis, including those reported earlier (2), was a lymphomatous involvement of many of the visceral organs. The frequency of tumors, and the distribution of the type of involvement in the 6 organs most often found grossly tumorous, are

RPL 18, 20, and 21, the tumor involvement was primarily extravascular with a few tumor cells within the sinusoids and veins. These tumors consisted primarily of lymphocytes, lymphoblasts or hemocytoblasts, and intermediate forms. Cells with 2 or 3 nuclei were also found as well as cells in various stages of degeneration.

After implantation with tumor cells the birds usually exhibited a gradual decrease in the number of lymphocytes in the circulating blood. In the terminal stages there was a sharp rise in the number of lymphoblasts, hemocytoblasts, and degenerated forms. Chickens with tumors produced by

TABLE IV: GROSSLY VISIBLE FOCAL AND DIFFUSE INVOLVEMENT OF THE VISCERA AFTER INOCULATION WITH CELLULAR OR CELL-FREE PREPARATIONS

	No. of visceral positive cases	Liver		Spleen		Kidney		Gonad		Pancreas		Mesentery or Peritoneum	
		focal	dif.	focal	dif.	focal	dif.	focal	dif.	focal	dif.	focal	dif.
STRAIN RPL 18													
Cellular pas., Series A	28	29	71	28	46	25	25	3	33	7	25	39	0
Cellular pas., Series B	97	39	58	14	68	11	20	10	18	7	15	21	7
Filtrate inoculations	41	43	56	34	61	7	25	0	12	0	0	0	2
STRAIN RPL 19													
Cellular pas., Series A	24	29	70	12	62	4	50	12	0	17	4	0	0
Cellular pas., Series C	41	15	80	5	71	0	22	2	7	0	3	7	5
Filtrate pas., Series B	91	9	89	2	80	0	13	11	9	1	1	15	2
Filtrate pas., Series D	53	30	70	8	85	0	6	4	0	0	0	0	0
STRAIN RPL 20													
Cellular pas., Series A	43	53	37	28	28	40	28	21	26	13	0	18	0
Cellular pas., Series B	30	44	53	3	67	23	40	27	13	10	0	3	0
Filtrate inoculations	41	35	61	37	54	34	56	5	39	0	2	0	5
STRAIN RPL 21													
Cellular pas.	50	40	60	12	74	32	28	6	41	6	10	12	6
Filtrate inoculations	20	70	25	25	70	65	25	5	25	5	15	10	20

presented in Table IV. In all the series of inoculations for the 4 strains, the liver was most often tumorous, followed closely by the spleen, and then by the kidney and gonad. At necropsy, the parenchymatous organs appeared to be diffusely involved in the majority of cases; however, upon microscopic study, most of these tumors were found to be made up of small focal areas. The localization of tumor tissue in areas of about 0.5 mm. to 10 mm. in diameter occurred frequently and was the predominant finding in the liver and spleen of birds in some inoculations.

Osteopetrosis similar to that described by Jungherr and Landauer (16); and Burmester, Prickett, and Belding (3) appeared in certain of the filtrate inoculations of Strains RPL 18 and 21. A large amount of necrosis with ensuing fibrosis was noted in tumorous livers of Strain RPL 20, a smaller amount in RPL 21, and only occasional evidence of this process was seen in Strains RPL 18 and 19.

In the livers of birds inoculated with Strains

filtrates usually had a slight decrease in the number of lymphocytes in circulation with a mild but definite rise in granulocytes and hemocytoblasts.

The main characteristic distinguishing Strain RPL 19 from other lymphoid tumor strains is the location of the tumor cells principally within blood vessels, resulting in a diffuse distribution of the tumor tissue. The tumor consisted primarily of hemocytoblasts with many intermediate lymphocytic and granulocytic forms. A second feature of Strain RPL 19 was the high incidence of hemangiomatosis that occurred in one series of filtrate passages of this strain. The hemangiomas appeared to be of the cavernous type and were located primarily in the loose connective tissue. With two exceptions, all chicks with hemangiomas also had grossly visible tumefaction of one or more of the visceral organs.

DISCUSSION

Transmissibility.—Several transplantable avian lymphoid tumor strains have been described (1, 2,

12, 22). With the exception of the results obtained by Duran-Reynals (9), who was unable to transplant successfully any of 12 tumors from cases of visceral lymphomatosis, the more recent work indicates that the procurement of highly malignant lymphoid tumor strains is not difficult. Burmester and Prickett (2) obtained transplants with tumors from 4 of 6 original donors and were able to propagate strains originating from 3 different cases of visceral lymphomatosis. In a later series (5) tumors from 8 of 10 cases of visceral lymphomatosis were successfully transplanted. Results of propagating 4 of these tumors in serial passage have been presented in this report. The incidence of tumors, average survival period, and passage interval after the first few transfers, were similar to those obtained with the tumor Strains RPL 14, 15, 16, and 17 reported on previously (2). It would appear that when cellular preparations of tumors, obtained after the first few passages, are injected into chicks 2 to 75 days of age, a high incidence of tumors may be expected within a period of 4 weeks. It should be recognized that almost all results reported herein are based on inoculations in one inbred line of chickens (line 15).

Until recently there was little evidence of the presence of filtrable agents in lymphoid tumors. Burmester and his associates (3, 6) demonstrated the presence of and propagation in serial passage of a filtrable tumor-inducing agent in a lymphoid tumor strain. However, the significance of these findings relative to the transmission of visceral lymphomatosis was questioned by some because of the following considerations. Although the donor originating this tumor strain had all the characteristics of a case of visceral lymphomatosis (lymphocytoma) it was not diagnosed as such by the investigator (22) and it was thought that the resulting transplantable strain was an unusual one (23). In addition, the tumor had been transplanted more than 200 times in chickens, some of which may have been harboring concurrently one or more disease agents.

Significant evidence that filtrable tumor-inducing agents were present in cases of naturally occurring visceral lymphomatosis was presented by Burmester and Denington (5). They found that cell-free preparations, from 5 of 10 such cases tested, produced similar tumors in chickens injected by the intraperitoneal route. The tumor from another (G736U2) of the 10 original cases was later found to carry an active filtrable agent (Table III).

More conclusive evidence of the filtrability of the agent or agents in lymphoid tumor strains recently derived from cases of visceral lymphomatosis

was obtained with Strains RPL 18, 20, and 21. The reasons for the omission of results with Strain RPL 19 from evidence for the transmission of lymphomatosis by a filtrable agent are presented later in this section. All strains propagated by cell transplantation were found to carry a filtrable agent or agents inducing a high incidence of lymphomatous tumors of the viscera indistinguishable from tumors in naturally occurring visceral lymphomatosis.

Variability in activity and manifestation.—There was some variation in the activity of these filtrates. In the third-passage filtrability test of Strain RPL 18, only 2 of 14 chicks developed visceral tumors, whereas the incidence was high in the first, second, and fourth passage tests. The use of different genetic lines of chicks for the third passage test may possibly account for this difference, however it does not explain the variation obtained with strains RPL 20 and 21, since only line 15 chicks were used for all inoculations with these strains. Filtrates and cell-free preparations of Strain RPL 20 tumors induced a high incidence of neoplasia in passages 1, 3, 5, and 6, but in passage 2, none of 7 birds injected with Mandler filtrate developed tumors. That the tumor was highly malignant is indicated by the result that 8 of 9 birds that received the cell suspension died in an average of 21 days with visceral tumors. No evidence of neoplasia was found in 20 chicks injected with a cell-free preparation of the tumor originating Strain RPL 21, but filtrates prepared from tumors of the fifth cellular passage of this strain produced a high incidence of visceral tumors and osteopetrosis.

This variation in activity of filtrates from lymphoid tumor strains may be due to a variation not only in the amount and virulence of the agent but also to a variation in the presence or activity of a "neutralizing" or "masking" agent. It has been amply demonstrated that the Rous tumor virus may be completely neutralized by an antibody-like agent which may be separated by centrifugation (7, 8, 20). A similar phenomenon apparently operates with the Shope papilloma virus (11, 17, 18) and has been suggested as a possible explanation for the variation in transmission obtained with tumor filtrates of different cases of naturally occurring visceral lymphomatosis (5).

The high speed centrifuged fraction of Strain RPL 21 tumor produced a higher incidence (100 per cent) of tumors than did the inoculum filtered through a Mandler candle (incidence of 87 per cent, Table III). These results indicate that an appreciable amount of the active agent was sedimented by the centrifugal speed used. Similar re-

sults have been obtained (4) with the lymphoid tumor Strain RPL 12. The occurrence of 4 cases of neurolymphomatosis among the 13 chickens inoculated with centrifuged sediment, while there were none among 15 injected with the filtrate, is difficult to explain.

A variation in the type of tumors obtained upon inoculation with cell-free preparations was also a prominent feature of tumor strains reported herein. A high incidence of osteopetrosis was obtained after inoculation with cell-free preparations of Strain RPL 18 (first passage) and Strain RPL 21 (sixth passage). One case appeared in another strain (RPL 19, first passage). A significant incidence of neurolymphomatosis was obtained in only one inoculation. Of the 13 chicks injected with the high speed sedimented fraction of Strain RPL 21 (Table III), 4 developed symptoms and gross lesions typical of neurolymphomatosis, whereas none of a total of 34 control chicks of the same stock developed similar symptoms or lesions.

The primary characteristic of the 4 strains was the occurrence of a high incidence of visceral tumors. The tumor tissue of Strains RPL 18, 20, and 21, was located extravascularly, whereas that of RPL 19 was located primarily intravascularly. Tumor tissue in the liver of the original donor (G124E) of Strain RPL 19 was located primarily extravascularly with some indication of endothelial activity and the presence of a few tumor cells in the sinusoids (5). The recipients of the first passage showed a wide variation in the location of tumors, from those primarily extravascular to those mostly intravascular. In all subsequent passages of Strain RPL 19, including the cellular and cell-free series, the neoplastic cells were located primarily within the formed vasculature of all tumorous organs. This characteristic distinguishes it from tumors usually found in visceral lymphomatosis (10, 15) and from other lymphoid tumor Strains RPL 12, 14, 15, 16, 18, 20, and 21 (2, 22).

The "latent" or incubation period of filtrates of Strain RPL 19 was found to be much shorter than that for similar preparations of other strains. When cellular inoculum of any of the 4 strains was used the tumors developed in a short time and death occurred early (Table I). This was particularly evident after a few passages in series. The average survival period of birds that died with visceral tumors after having been inoculated with filtrates of Strains RPL 18, 20, or 21, was much longer (Table III—average of 128 to 162 days) than for the chicks in the cellular passage of the same strains (Table I—average of 5 to 27 days for later passages). These results are in agreement with those

reported earlier (3, 6) for the lymphoid tumor Strain RPL 12, but are in contrast with those obtained with RPL 19. Filtrates of this strain produced death with tumors in a much shorter time (21 to 43 days for the later passages, Table II). The period was only about twice as long as for cellular inoculum, in comparison with about 10 times for the other strains.

The relatively short "latent" or incubation period of filtrates of Strain RPL 19 and the intravascular location of tumor tissue, are features usually associated with the easily transmissible erythrogranuloblastosis. Although most of the tumor was made up primarily of hemocytoblasts with intermediate lymphoid forms, immature cells of the erythrocytic and granulocytic series also were seen in the tumor and peripheral circulation. Because of these characteristics one may suggest that at least one of the agents of this strain is similar to the transmissible agent of fowl leukosis described by many investigators. (*See* review of Olson, [21]).

The occurrence of hemangiomatosis in tumor strains derived from cases of visceral lymphomatosis appears to be unusual. Its occurrence at such a high rate has thus far not been reported. No evidence of it was found in chickens inoculated with the several RPL tumor strains described earlier (2) and of the strains described in this report, only one bird aside from those inoculated with Strain RPL 19 developed hemangiomas. These tumors appeared in the birds of several inoculations of the second passage of the latter strain. Their incidence was increased to a high percentage in a series (A) of passages made with filtrates prepared from tumorous livers of donors having hemangiomas. In another series (B) the occurrence of angiomas was eliminated in 3 passages made with filtrates prepared from tumorous livers of donors without evidence of angiomas (Table II). In both series almost all birds had a lymphoid involvement of the viscera, primarily the liver and spleen. Furth (12, 13) obtained endotheliomas with myelocytomatosis or hemocytoblastosis after inoculation with filtrates of his Strain 2. Since hemangiomas also are due to a neoplastic proliferation of the endothelium, it may be suggested that agents of Strain 2 and of RPL 19 producing endothelial tumors were similar, if not identical, though there are consistent morphological differences which are not accounted for by this simple explanation. Furth thought that the various manifestations which he obtained with Strain 2 were due to a single agent. Results obtained with Strain RPL 19 indicate that the tumor originating this strain contains several tumor agents or variants. Manifestations of an

intravascular lymphoid leukosis agent, though not evident in the original donor, appeared in the first passage and was a dominant feature in all other inoculations. Manifestations of a filtrable hemangiomatosis agent appeared in the several passages and were increased (series A) and decreased (series B) by selection. Since the original donor appeared to be typical of visceral lymphomatosis, and a filtrable agent that reproduces extravascular lymphomatous tumors has been demonstrated in similar tumors of visceral lymphomatosis, the original tumor of RPL 19 may also have contained a similar agent but one of low virulence or one that was partially "neutralized."

The occurrence of osteopetrosis and neurolymphomatosis only in certain strains and certain inoculations, together with the results obtained with Strain RPL 19, would favor the view that the various forms of the avian leukosis complex (14) were caused by different agents rather than by a single multipotential agent. Changes in a parent agent, as by mutation, may also be suggested as an explanation for the transmission results that have been reported here and elsewhere.

The variation in pathological manifestations (visceral lymphomatosis [extravascular], intravascular lymphoid leukosis, neurolymphomatosis, osteopetrosis, hemangiomatosis) and transmission obtained in inoculations with cellular and cell-free preparations of tumors from cases of naturally occurring visceral lymphomatosis (5) and in subsequent serial passage inoculations would suggest that a variety of agents or variations of the "parent" agent were present in the original tumor. The possibility that contaminants were acquired during some of the passages is not very likely since the variations were obtained within a few passages of the original donor, the birds were maintained under quarantine, and aseptic technic was used when handling the inoculum.

If several agents were present in the original donor, part of these must have been "masked" or present in an inactive form, since the original donors showed evidence of only visceral lymphomatosis or, in addition, gross or microscopic lesions typical of neurolymphomatosis. That some or all of the agents were present in an inactive or "masked" form has already been suggested in this report and elsewhere (5). Thus evidence has been presented suggesting that many cases of naturally occurring lymphomatosis, though showing only one form of the disease, may carry several tumor agents, any number (possibly all) of which may be in "masked" or inactive form but under favorable circumstances may become visible.

SUMMARY AND CONCLUSIONS

1. The characteristics of 4 tumor strains, RPL 18, 19, 20, and 21, recently developed from cases of naturally occurring visceral lymphomatosis, were studied during their serial passage with cellular inoculum and in numerous passages with filtered preparations.

2. The injection of cellular suspensions into the peritoneal cavity of chicks 2 to 75 days of age produced regularly a high incidence of the disease with a lymphomatous involvement of the liver, spleen, kidney and other organs of the viscera. The tumorous birds almost invariably died within 4 weeks and passages were made in 5 to 15 days after inoculation.

3. Tumor preparations or plasma from tumor-bearing birds, rendered cell-free by centrifugation or filtration through bacteria-retaining filters, also produced a great number of cases with visceral tumors. None of the 34 non-inoculated controls developed tumors during the experimental period of 200 days.

4. The tumor cells in cases of Strains 18, 20, and 21, similar to naturally occurring visceral lymphomatosis, were located primarily extravascularly and filtrates of these strains had a comparatively long latent period (average survival period of 116 to 162 days).

5. In addition to the visceral tumors, cell-free preparations of Strains RPL 18 and 21 also produced osteopetrosis in 4 of 7 inoculations in proportions of 5 to 77 per cent.

6. The tumor cells in cases of Strain RPL 19 were located primarily within blood vessels, and filtrates of this strain had a comparatively short incubation period, features usually associated with transmissible erythrogranuloblastosis.

7. In addition to the visceral tumors in birds of Strain RPL 19, the occurrence of hemangiomatosis was increased to 88 per cent in one series of passages and was reduced to zero in other series by the selection of appropriate donors.

8. Conclusive evidence is presented to show that tumors of visceral lymphomatosis may be reproduced by material which will readily pass through bacteria-retaining filters. This agent or agents may be propagated in serial passage by the inoculation of healthy chicks with cellular or cell-free preparations.

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Cytochemical Studies of Normal and Tumor Mast Cells in Tissue and *in Vitro**

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Since the first description of spontaneous mast cell tumors (mastocytomas) of dogs (2), subsequent studies have been concerned with the morphology and behavior of the neoplastic mast cells in tissue culture (11). Chemical preparations of these tumors have demonstrated considerable quantities of an anti-coagulating substance that is presumably heparin (10). The present study demonstrates the presence of lipids, cytochrome oxidase, and acid and alkaline phosphatases in normal and tumor mast cells and of tumor mast cells grown in tissue culture for several weeks.

METHODS

Normal tissue mast cells were examined in sections of rectum and in whole mounts of mesentery obtained from healthy dogs. The tumor mast cells were obtained from a solitary benign mastocytoma located in the subcutaneous tissue of a dog. Imprints were made of the fresh tumor and stained with Wright-Giemsa. Tumor fragments were planted in 1 drop of chicken blood plasma, 2 drops of dog serum and 1 drop of chick embryo extract for cultivation *in vitro*. The cultures were fixed *in toto* and not removed from the cover glass, thus avoiding disturbance of the delicate new cells radiating into the culture medium.

The lipids were studied in tissues fixed in formolcalcium-cadmium, stained with Sudan IV and Sudan Black B according to the procedure of Baker (1). The Smith-Dietrich test for phospholipids

was also used on all tissues. The M-Nadi reagent for "stabile" cytochrome oxidase was applied to fresh and formalin-fixed tissue (7). Alkaline phosphatase was demonstrated by Gomori's method (5) and acid phosphatase by Wolf, Kabat and Newman's modification (12) of Gomori's method (6).

RESULTS

Table I summarizes the enzyme and lipid content found in uncultured normal and tumor mast cells, and in tumor mast cells cultivated *in vitro*. The phosphatases were present in the form of cyto-

TABLE I: CYTOCHEMICAL OBSERVATIONS ON NORMAL AND TUMOR MAST CELLS OF DOGS
SUBSTANCES DEMONSTRATED IN CELLS

| Mast cells | Alkaline phosphatase | | Acid phosphatase | | "Stabile" cytochrome oxidase | Lipids |
	As cytoplasmic granules	In nucleus	As cytoplasmic granules	In nucleus	As cytoplasmic granules	In mast granules
Normal cells not cultured	X (Fig. 1)	....	X (Fig. 3)	....	X	X (Fig. 5)
Tumor cells not cultured	X	X	X	X	X	X
Tumor cells cultured *in Vitro*	X (Fig. 2)	X (Fig. 2)	X (Fig. 4)	X (Fig. 4)	X	X (Fig. 5)

plasmic granules; however, it was difficult to ascertain whether or not these corresponded to the mast granules. Some cultured cells contained a paranuclear granule-free area (11). When stained for acid phosphatase this region revealed a delicate black reticulum (Fig. 4) which is suggestive of the Golgi apparatus revealed in other tissue cells (3, 4) when they are stained for phosphatases.

DISCUSSION

The observations in Table I reveal that both normal and tumor mast cells contain acid and alkaline phosphatases in their cytoplasm (Figs. 1-4). In tumor mast cells, however, both enzymes were found in the nucleus as well (Figs. 2, 4). In normal mast cells of the rat, Noback and Montagna (9) demonstrated alkaline phosphatase only in the cytoplasm. The cultured tumor cells evidenced phosphatase content identical with the original tumor cells, indicating that the former maintain their phosphatase constituents despite the changes in cell morphology which occur in tissue culture. Controversy exists concerning the lipid character
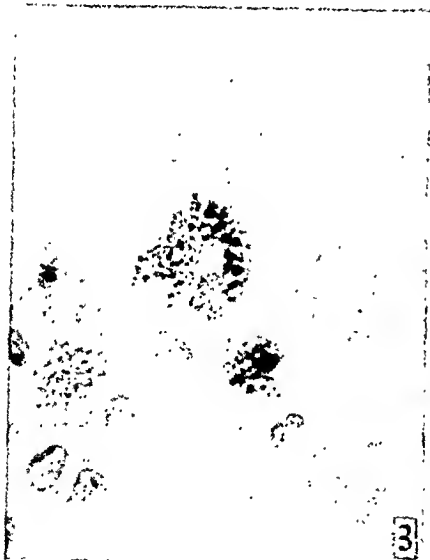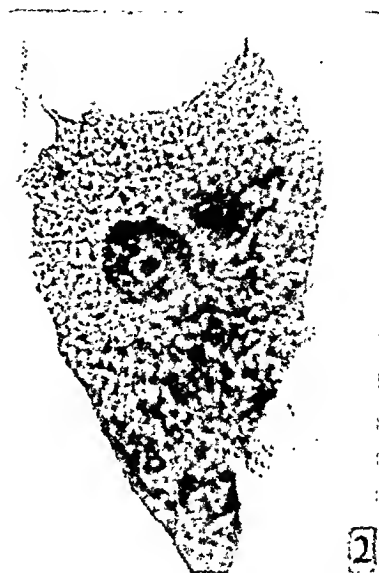
FIG. 1.—Normal mast cells from the interstitial tissue of the anal sacs of dog. Alkaline phosphatase reaction restricted to the cytoplasm. Counterstained with paracarmine. Mag. × 970.

FIG. 2.—Tumor mast cell cultured for 4 weeks *in vitro* with strong alkaline phosphatase reaction in the nucleus and in the cytoplasm. Mag. × 970.

FIG. 3.—Normal mast cell from the interstitial tissue of the anal sacs of dog demonstrating acid phosphatase reaction confined to the cytoplasm. Counterstained with paracarmine. Mag. × 970.

FIG. 4.—Tumor mast cell cultured for 4 weeks *in vitro* showing strong acid phosphatase reaction in both the nucleus and cytoplasm. The structure adjacent to the nucleus is probably the Golgi apparatus. Mag. × 970.

of the mast granules of normal mast cells (8). In our material, Sudan black B revealed lipid granules in all categories of mast cells examined (Fig. 5). Sudan IV, however, gave negative results. The distribution of the lipid droplets appeared to coincide both qualitatively and quantitatively with the distribution of the mast granules. Excellent stain-

ing of the lipid granules were obtained despite previous immersion of the tissues in alcohol, acetone, chloroform, and other lipid solvents at 60° C. for 48 hours. When mast cells fixed in Baker's formolcalcium-cadmium are stained by the Smith-Dietrich method, vaguely distinguishable black granules can be seen in the cytoplasm. These facts give pre-
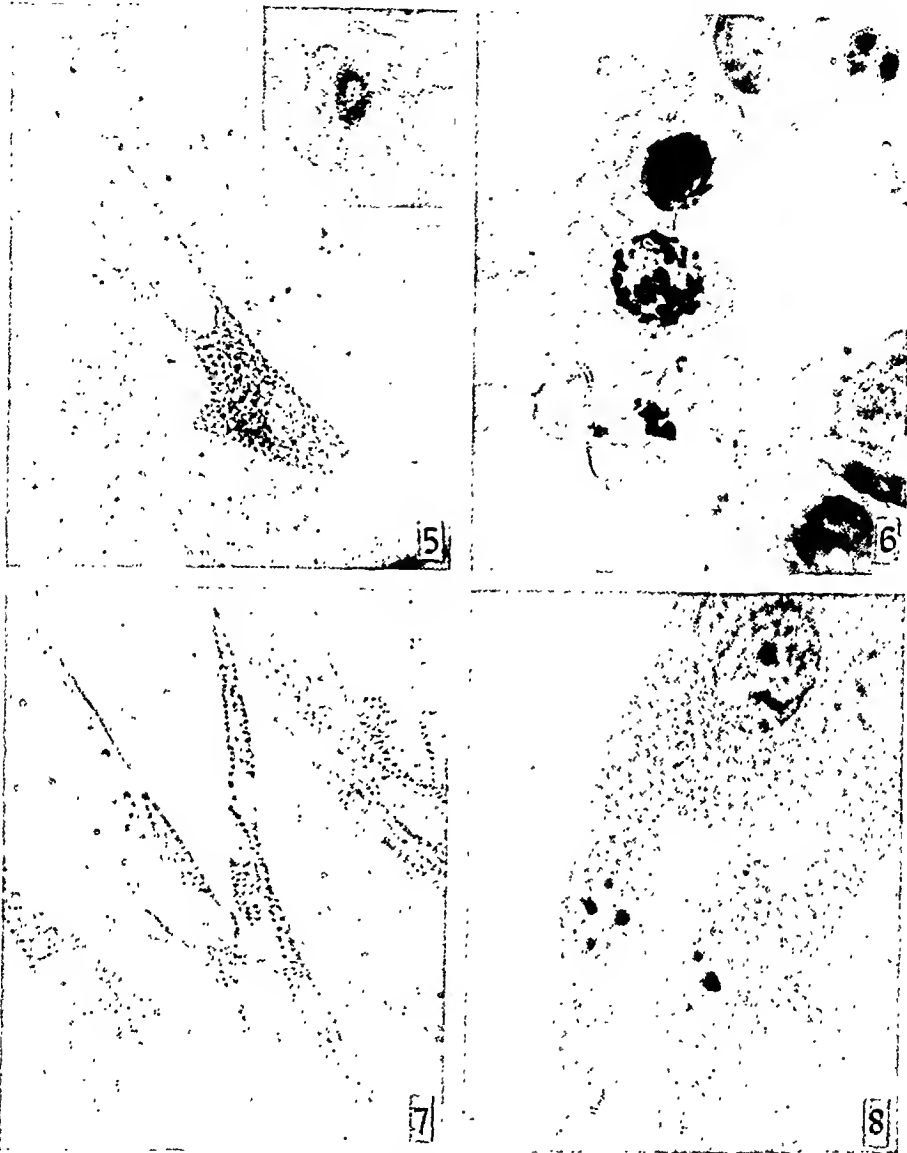
FIG. 5.—Normal mast cell (inset) from interstitial tissue of the anal sacs of dog depicting lipid granules. Sudan black. Mag. $\times$ 440.

The larger cell with tenuous processes is a cultured tumor mast cell with lipid granules. Sudan black. Mag. $\times$ 440.

FIG. 6.—Imprint preparation of tumor mast cells stained with Wright-Giemsa. Mag. $\times$ 970. This cell type *in vitro* resembles morphologically those depicted in Figs. 2, 4, 5, 7 and 8.

FIG. 7.—Living unstained tumor mast cells cultured 6 weeks *in vitro*. Mag. $\times$ 440.

FIG. 8.—Tumor mast cells cultured 6 weeks *in vitro*; with large cytoplasmic granules. Iron hematoxylin. Mag. $\times$ 970.

sumptive evidence that the lipid substances in the mast cells of the dog consist of lipins.

Opinions differ concerning the presence or absence of oxidase in the mast cell granules (8). In our material, "stabile" cytochrome oxidase was demonstrated in the mast granules of uncultured normal and tumor cells, and of cultivated cells *in vitro*.

SUMMARY

Histochemical studies of normal and tumor mast cells, and of tumor mast cells cultivated *in vitro*

reveal that the mast cells in all these categories contain lipids, cytochrome oxidase, and acid and alkaline phosphatases in their cytoplasm. The tumor cells, in addition, contain alkaline and acid phosphatases in their nuclei.

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The Elimination of 3, 4-Benzpyrene from a Human Being after Intravenous Injection

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The metabolism of 3,4-benzpyrene in fowls, rats and mice has been outlined by Peacock and his co-workers (12, 9, 5) and during subsequent years these and other investigators (1-3, 6-8, 10, 13-15) have succeeded in identifying different metabolites of 3,4-benzpyrene.

In this laboratory an opportunity has been afforded of investigating roughly the metabolism of 3,4-benzpyrene in a human being. It has not been possible to perform a complete quantitative analysis, partly on account of the considerable variation both quantitatively and qualitatively in the content of interfering substances as a result of the patient's intake of food and medicine, and partly because the amount of excretion made purification and part-isolation impossible in a reasonable time. The 3,4-benzpyrene used in this investigation was synthesized by Professor H. Lund, of the University of Aarhus.

MATERIAL AND METHOD

The patient was a 33 year old man, suffering from myeloid leukemia; he was treated according to Engelbreth-Holm and Stamer (11). Over a period of six weeks 5 injections were given amounting to a total of 4.6 gm. of 3,4-benzpyrene—1 injection of 600 mgms. and 4 injections of 1 gm. 3,4-benzpyrene suspended in 100 cc. of 2 per cent Postonal-water. Each injection lasted approximately 3 to 5 minutes. The patient weighed 70 kilos. The excretions were kept in darkness and collected morning and evening.

Urine.—This was shaken for 2 hours with a double volume of benzene, after which the benzene was dried over sodium-sulphate and passed through a column (25×5 cm.) of aluminum oxide. After development with pure benzene 3 main zones were seen in ultraviolet light: At the top a light yellow zone (A); below this and moving slowly downwards, a slightly darker yellow-green zone (B) and

at the bottom of the column and moving out into the filtrate a bluish violet zone (C).

After cutting and repeated chromatography, zone A, dissolved in ethanol, showed only non-characteristic absorption. (All absorption measurements were carried out on a Beckman photo-electric Spectrophotometer). Zone B, after cutting and repeated chromatography and, finally, dissolution in ethanol, showed a strong blue fluorescence, which became more greenish after the addition of alkali. The absorption spectrum for zone B is given in Fig. 1, A. The graph, which represents the average of 10 single determinations, shows maxima at 361, 380, 395 and 418 mμ.

The benzene filtrate containing zone C was evaporated to dryness under reduced pressure in a N$_2$-atmosphere. The absorption curve for this zone showed the presence of unchanged 3,4-benzpyrene.

Zones B and C were present in the chromatogram of urine 3 hours after injection; the zones were most pronounced for 1 to 2 days after the injection, but had nearly disappeared in 4 or 5 days. It was not possible, judging from the chromatogram, to see any difference in the relative distribution between the two zones.

The shaking with benzene did not remove all the fluorescent material from the urine, neither did continuous extraction with ether for 72 hours. Therefore, shaking with benzene being the more convenient, this method was used.

Feces.—These were extracted with acetone until the extract was only slightly yellow, then the acetone extract was concentrated to approximately a tenth of the volume under reduced pressure and in a N$_2$-atmosphere. After adding 500 cc. 1 N HCl, the solution was kept at 100° for 15 minutes with a current of N$_2$ passing through it. After cooling, the mixture was extracted with benzene, dried with sodium-sulphate and passed through a column (25 ×5 cm.) of aluminum oxide. It was then developed with pure benzene.

In ultraviolet light a brownish black zone 2 to 3 cm. wide was seen at the top and just below this a yellow-greenish zone, which moved slowly downwards during the development. This zone exhib-
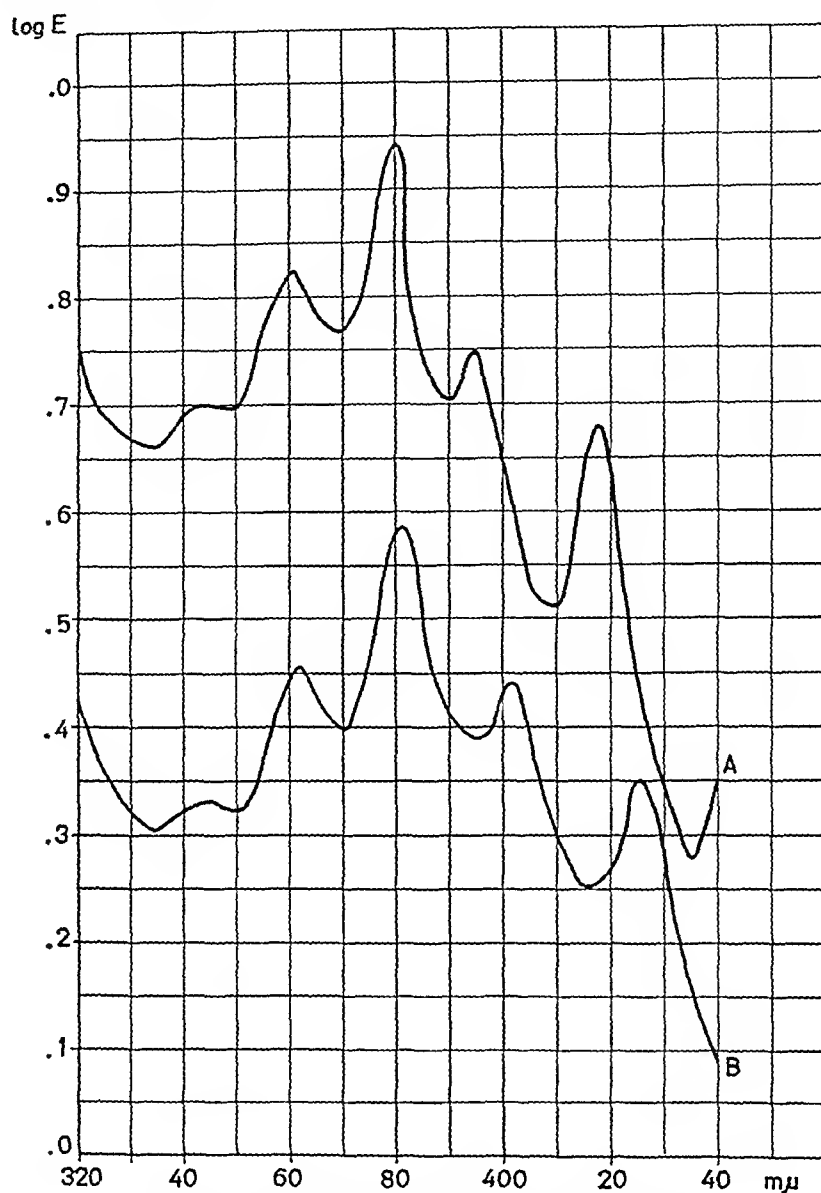
Fig. 1.—A = absorption spectrum of eluate from yellow
zone from urine. B = absorption spectrum of eluate from
yellow zone from feces.

ited, after repeated chromatography, a bluish
fluorescence in ethanolic solvents but changed color
after the addition of alkali. The absorption curve
for this zone is given in Fig. 1, B. The graph rep-
resents the average of 12 separate determinations,
and illustrates maxima at 362, 381.5, 402 and 425
mμ. As in urine, a bluish zone was seen moving
downwards and into the filtrate. The presence of
unchanged 3,4-benzpyrene was detected in this
zone after purification by repeated chromatography.

Beneath the yellow zone a reddish fluorescence

sometimes appeared, which red zone moved down-
wards more quickly than did the yellow one, and
was present only when the feces had remained
rather long in the bowels. On one occasion the
patient was constipated for 3 days after an injec-
tion. After a water enema the patient defecated 5
times in the next 12 hours. In the chromatogram
of the first defecation a broad, very intensive red
zone was seen; in the chromatogram of the next
two the red zone was narrower, and in the chro-
matogram of the last 2 defecations no red zone
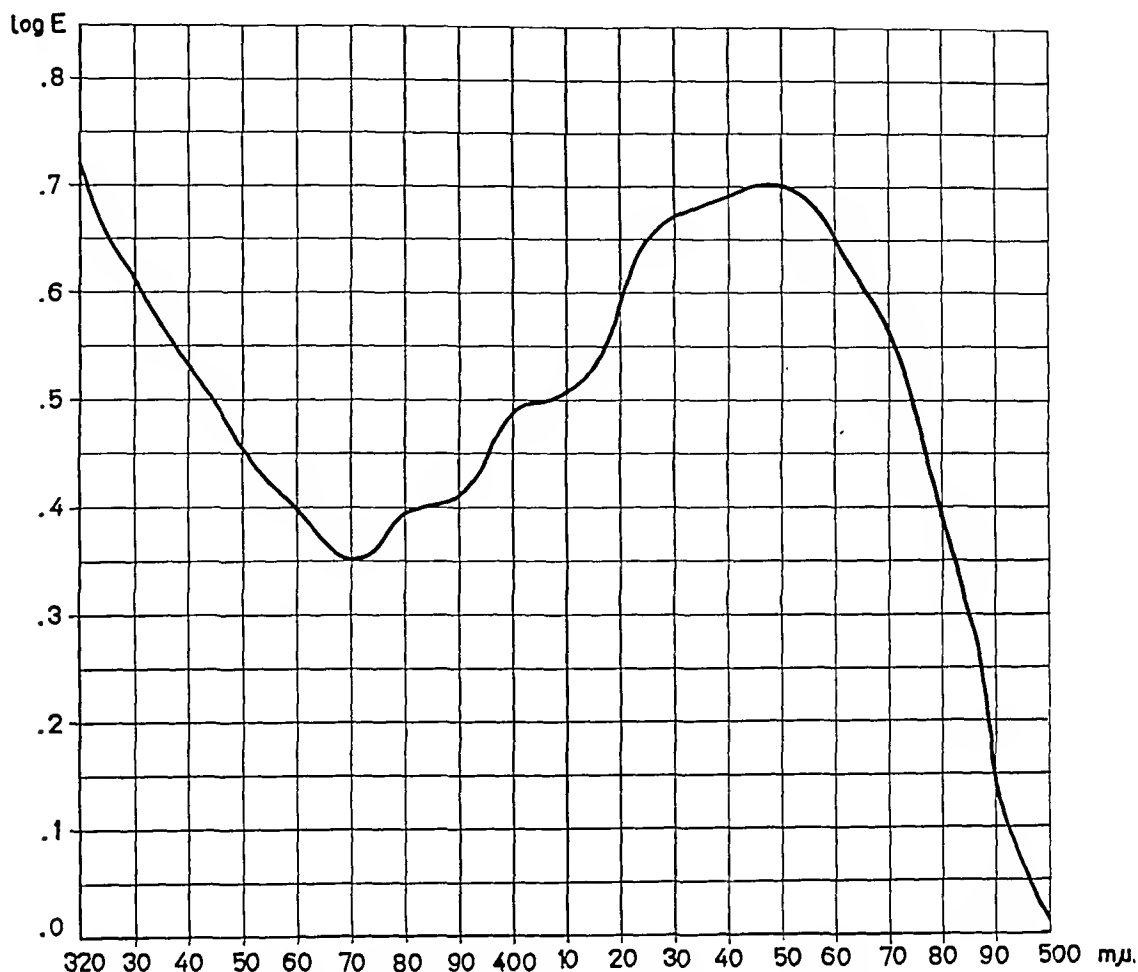
FIG. 2.—Absorption spectrum of eluate from red zone from feces.

was visible. Both the yellow and blue zones were present in all 5 defecations. Fig. 2 represents the absorption curve of the red zone dissolved in ethanol. The blue fluorescent filtrate showed the presence of unchanged 3,4-benzpyrene.

Blood.—Five cubic centimeters of blood taken 3 hours after injection were immediately extracted with 25 cc. of acetone and treated as described under *Feces*. In the chromatogram a very narrow yellow fluorescent zone and a very broad blue fluorescent zone were noted. Following repeated chromatography, the absorption curve from the blue zone indicated the presence of unchanged 3,4-benzpyrene. After evaporation of the eluate containing the yellow zone, the residue gave a positive ninhydrine test.

A further 5 cc. of blood was taken 3 hours after injection but not extracted with acetone until 6 hours later. In other respects it was treated in the same way as the first 5 cc. In this chromatogram there was a distinctly broader yellow fluorescent zone than in the first.

Skin fluorescence.—Before, during, and following an injection the fluorescence of the skin was examined spectroscopically. Fig. 3 shows the photometer tracings of (a) pure benzpyrene, (b) the fluorescence of the skin 1 hour after injection (c, d, and e) the fluorescence of the skin 2¼, 6 and 25 hours after injection, the last-named being identical with the fluorescence of the skin before the injection. As it will be seen, metabolites of 3,4-benzpyrene are not detectable in these fluorescence spectrograms.

Organs.—The patient died from his leukemia 10 days after the last injection and the organs were removed 12 hours after death. No "foreign"

405   427 436   455   510 mµ
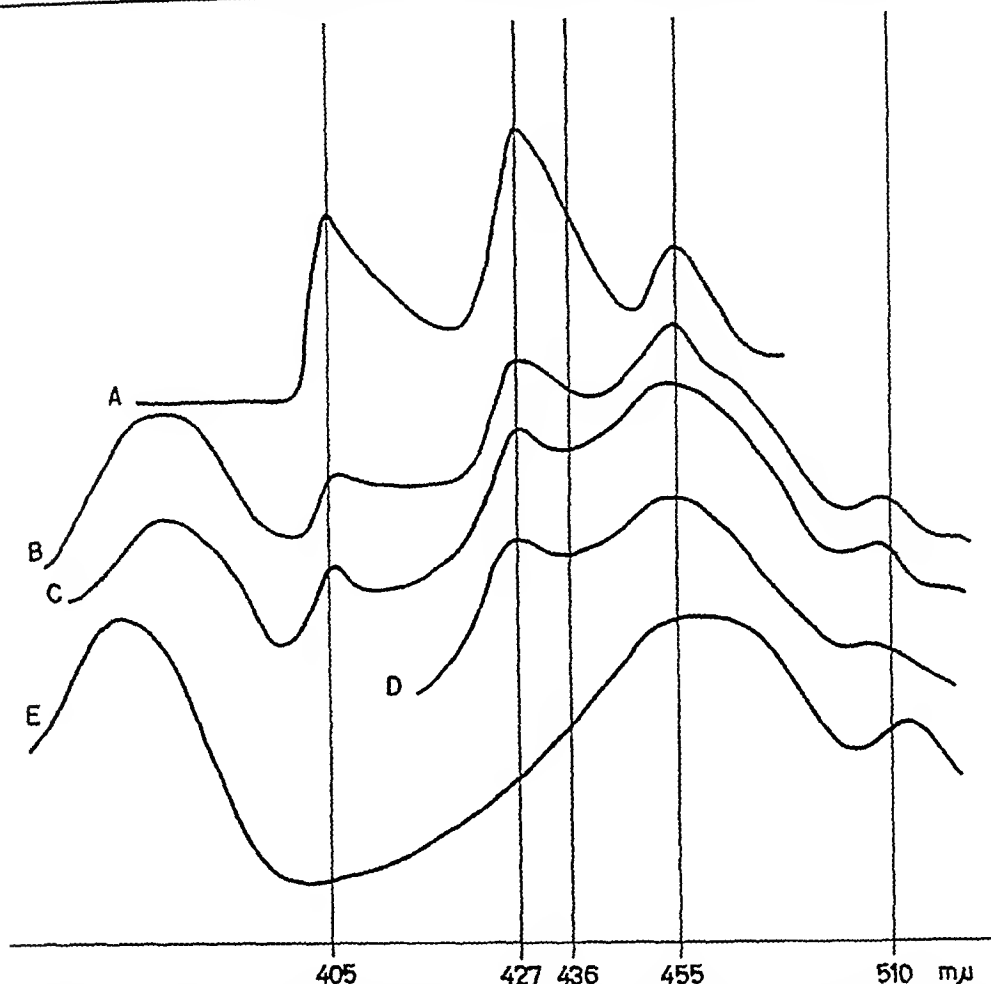
FIG. 3.—Photometer tracings of fluorescence spectra of (A) pure 3,4-benzpyrene, (B) skin 1 hour after injection, (C) skin 2½ hours after injection, (D) skin 6 hours after injection, (E) and skin 25 hours after injection (identical with fluorescence spectra of skin previous to injection).

fluorescence was noticed by macroscopic inspection in ultraviolet light in the brain, lungs, spleen, liver, kidneys and fat. In the purified acetone extract (treated as described under *Feces*) no absorption curve characteristic of 3,4-benzpyrene or of its derivatives was obtained.

Photosensitivity.—The eluate from the yellow zone from urine and feces shows, as described by numerous other investigators, a marked instability towards ultraviolet light. To establish a numerical expression for this photosensitivity a sample of the ethanolic eluate from the yellow zone of feces and a benzpyrene solution (ethanolic) with approximately the same extinction value at 385 mµ as had the eluate at 381.5 mµ, were exposed simultaneously and at the same distance to the light of a super high-pressure water-cooled mercury lamp and the decrease in extinction values at 381.5 and 385 mµ were measured. Fig. 4 shows the decrease in extinction expressed as a percentage of the original extinction.

As stated before it was not possible to perform a quantitative analysis, but to obtain a rough idea of the amount of metabolites excreted, a calculation, adopting the extinction 1.0 in a cell 1 mm. in length, corresponding to the amount of F derivatives obtained from 0.01 mgm. 3,4-benzpyrene, as given by Weigert and Mottram (14). was carried out on all the purified extracts obtained from feces during the first 5 days after injection. It was not possible to obtain any characteristic absorption from the crude acetone extracts. The result was that F derivatives corresponding to 168 mgm. 3,4-benzpyrene were found. This figure is without doubt too low, owing to the serious losses during purification. A calculation of the amount of me-
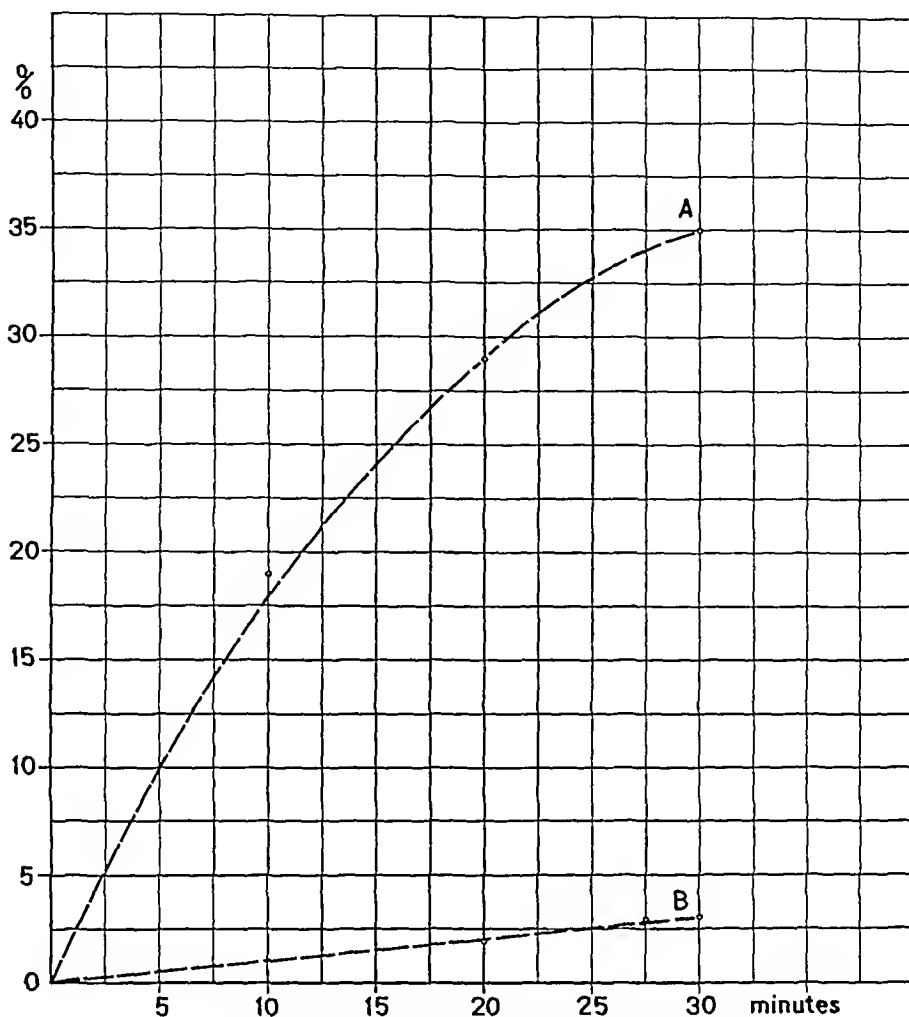
Fig. 4.—Decrease in extinction after ultraviolet irradiation. A = eluate from yellow zone from feces. B = 3,4-benzpyrene.

tabolites excreted in urine was not made as the extraction was by no means complete.

DISCUSSION

Weigert and Mottram (14, 15), have shown the presence of 4 different metabolites of 3,4-benzpyrene in the organism after application of 3,4-benzpyrene. The absorption curves obtained in this investigation are in very close agreement with the curves for F1 and F2 derivatives given by these investigators so that it would seem reasonable to conclude that the yellow zone from urine (Fig. 1, A) is identical with F1 and the yellow zone from feces (Fig. 1, B) is identical with F2(=8-benzpyrenol).

Thus, the evidence suggests that benzpyrene may be metabolized by man in the same way that it is in certain other species.

The resemblance between the absorption curves for F1 and F2 is very great, which leads to the suggestion that F1 and F2 are in reality the same 3,4-benzpyrene derivative but attached to different interfering substances. As to the exact nature of the possible common interfering substances one can only guess, but the finding of a positive ninhydrine test strongly supports the suggestion that the interfering substance may be of a protein nature. The assumption that the breaking down of an attachment between protein and 3,4-benzpyrene during the conversion of x-derivatives to F-derivatives is physiologically more likely than the assumption of a ring-opening and ring-closing (5). The finding of an increased protein (amino acid) elimination in the urine after intravenous injection of

carcinogenic hydrocarbons would support the hypothesis. and investigations of this sort are being carried out, the findings of which will be published at a future date.

CONCLUSION

1. The presence of F1 and F2 (=8-hydroxy-3,4-benzpyrene) in urine and feces from a human being after intravenous injection of 3,4-benzpyrene has been shown.

2. By spectroscopic examination of the fluorescence of the patient's skin after intravenous injection of 3,4-benzpyrene no metabolites of 3,4-benzpyrene were detected.

3. A positive ninhydrine test was found in the residue from the eluate of the chromatographically separated yellow zone from plasma.

4. No evidence of 3,4-benzpyrene or its derivatives was found in the organs 10 days after death.

SUMMARY

3.4-Benzpyrene was injected intravenously into a patient suffering from myeloid leukemia. The total amount injected was 4 to 6 gm. Compounds with absorption spectra identical with those given by other investigators for metabolites isolated from rats and mice were found in urine and feces. The fluorescence of the skin was examined spectroscopically before, during, and after injection. The photometer tracings of the fluorescence spectra showed only the presence of unchanged 3,4-benzpyrene.

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β—Glucuronidase Activity in Human Tissues
Some Correlations With Processes of Malignant Growth
and With the Physiology of Reproduction*

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It has been reported that elevated β-glucuronidase activity is present in cancer tissue excised from the primary lesion (3) or from metastases to lymph nodes and to other organs in the body (4). The most striking differences in enzymic activity as compared to the adjacent uninvolved tissues were observed in carcinoma of the breast with metastases to lymph nodes (5). In the present paper, a more complete survey has been made of . the β-glucuronidase activity of various malignant and benign tumors occurring in human beings. In addition some data concerning the β-glucuronidase activity of normal human ovary, uterus, and vagina are presented. The tissue findings are discussed in detail, especially in connection with theories of the function of β-glucuronidase in the organism and of carcinogenesis.

METHODS AND PLAN OF STUDY

The fresh tissue specimens removed at operation were dissected and the tumor was carefully separated from the uninvolved tissue. An attempt was made to select actively growing, non-necrotic tumor tissue and for its control whenever possible, a representative portion of the uninvolved tissue. The tissues were divided with a sharp razor blade, one portion was fixed in formalin for histological study and the other portion was assayed for β-glucuronidase activity (6). The diagnosis of each lesion reported was confirmed in every case by histological sections.

In Table I the β-glucuronidase activity of both malignant and benign lesions are grouped under the name of the organ involved. Table II represents a study of the enzymic activities of lymph nodes involved with metastatic carcinoma. In a few cases it was possible to study the primary lesion as well. Descriptions and photomicrographs of the histological sections of tissue from several of these cases are presented in the illustrations. In Table III, a list is presented of β-glucuronidase activities of tissue samples of the uterus, ovary and vagina of patients in various physiological states.

RESULTS

Six carcinomas and one fibrosarcoma of the breast showed 6- to 26-fold increases in β-glucuronidase activity as compared with the uninvolved tissue (Figs. 1, 2 and 7). In benign fibroadenoma and gynecomastia, the enzymic activity was relatively normal. In both chronic cystic mastitis and benign intraductal papilloma relatively high values were seen in uninvolved tissue. Moderate elevation in enzymic activity of the lesion was seen in 2 of these patients and a very high value in the third (Patient S.). These results should be interpreted with caution since in 2 of the specimens, cystic fluid, which contains glucuronidase (1), was included.

Six out of 7 carcinomas of the stomach and lower esophagus, showed unmistakable elevations in activity of 200 to 300 per cent (see Figs. 12 and 13). In the sixth instance (Patient Sa.), when compared with uninvolved esophagus the adenocarcinoma was higher in activity, but the difference was less marked when compared with uninvolved mucosa.

Of the 6 cases of adenocarcinoma of the colon (see also Figs. 16 and 17), 3 showed significantly elevated glucuronidase activity.

In five of these the comparison was made between tumor and uninvolved colon mucosa. This may not be a fair comparison as the tumor itself had involved both mucosal and muscular elements of the bowel and colonic mucosa is richer in glucuronidase activity than the muscle tissue. If 449 units

of glucuronidase is representative of a section through all coats of bowel (Patient B.), then the enzymic differences in all 6 colon carcinomas would become comparatively more striking. These same considerations would apply to the data reported on gastric tumors. In the lipoma, the activity was much lower than in the colon mucosa.

In the pancreas, no reliable comparison could be made because of the lack of uninvolved pancreatic tissue to serve as adequate control. However, in the islet cell adenoma somewhat higher activity was observed than in adjacent uninvolved pancreas.

The adenocarcinoma of the uterus showed ele-

TABLE I.—*β-Glucuronidase Activity in Human Neoplastic and Other Tissues*

Patient	Pathological diagnosis, lesion	Glucuronidase units* Involved	Uninvolved	Remarks
	BREAST			
C.	Carcinoma	890	168	
R.	Carcinoma	1,930	62	
M.	Carcinoma	945	....	
Mc.	Carcinoma	900	94	
K.	Carcinoma	3,650	143	
H.	Carcinoma	2,090	165	
D.	Fibrosarcoma	1,230	145	
K.	Benign fibroadenoma	393	325	
C.	Gynecomastia	267	....	
W.	Gynecomastia	96	....	
S.	Chronic cystic mastitis	16,100	770	Cystic fluid included in involved specimen analyzed in patients S, W.
W.	Benign intraductal papilloma and chronic cystic mastitis	1,870	....	
H.	Chronic cystic mastitis	1,810	1,042	Specimen consisted of cyst wall
	STOMACH			
B.	Adenocarcinoma of cardia	2,180	1,270	Uninvolved gastric mucosa
			650	Uninvolved esophageal mucosa
K.	Adenocarcinoma of cardia	1,510	750	
L.	Adenocarcinoma	3,180	940	
S.	Undifferentiated carcinoma	817	437	Mucosa and muscle coats
Sa.	Adenocarcinoma of cardia	853	486	Uninvolved esophageal mucosa.
			720	Uninvolved gastric mucosa.
R.	Adenocarcinoma	1,640	191	Mucosa and muscle coats
Ka.	Adenocarcinoma	2,320	636	Mucosa and muscle coats
		2,505		
	COLON			
N.	Adenocarcinoma	1,320	1,486	Uninvolved colonic mucosa
G.	Adenocarcinoma	2,770	1,628	" " "
O.	Adenocarcinoma	1,360	1,760	" " "
V.	Adenocarcinoma	900	1,930	" " "
M.	Adenocarcinoma	5,250	2,490	" " "
B.	Adenocarcinoma	827	449	Mucosa and muscle coats
N.	Lipoma	126	1,311	Uninvolved colonic mucosa
	PANCREAS			
M.	Spindle cell sarcoma	463	....	
B.	Adenocarcinoma	423	....	
E.	Adenocarcinoma	172	326	
B.	Benign islet cell adenoma	960	600	
	UTERUS			
H.	Adenocarcinoma	884	199	Myometrium
N.	Benign leiomyoma	405	1,370	"
H.	" "	165	198	"
C.	" "	425	173	"
	OVARY			
J.	Adenocarcinoma	1,410	....	
M.	Metastatic ovarian carcinoma to rectovaginal septum	2,350	605	Uninvolved rectal mucosa
			295	Uninvolved vaginal mucosa
C.	Recurrent adenocarcinoma	2,740	554	Mesentery
D.	Krukenberg from stomach	234	....	Mucoid acellular tumor
	OTHER ORGANS			
C.	Chondrosarcoma in chest wall	572	242	Uninvolved fibrous tissue
H.	Squamous cell carcinoma of penis	1,855	325	
Ca.	Transitional cell papilloma of bladder	1,850	....	
M.	Spindle cell sarcoma of lung	940	483	

* One glucuronidase unit is defined as 1 ugm. of phenolphthalein liberated from phenolphthalein mono-β-glucuronide per hour per gram of wet tissue at 38° under standard conditions (6).

vated activity whereas the benign leiomyoma showed less activity than normal in 2 of the 3 cases studied.

Ovarian carcinoma metastatic throughout the abdomen had high enzymic activity.

A high glucuronidase activity of the tumor compared to the adjacent uninvolved tissue was usually noted in the various other tumors from different parts of the body.

cinoma. These tissues were in no way involved with cancer. The non-surgical case was that of a patient who died 48 hours post-partum from pulmonary embolism where tissues were obtained at autopsy a short period later.

In the 2 younger patients, the tissues showed a much higher glucuronidase than the 3 who were in the post-menopausal state. In Patient N., the endometrium and endocervix showed a much higher

TABLE II: B-GLUCURONIDASE ACTIVITY IN PRIMARY LESIONS AND IN THEIR METASTASES TO LYMPH NODES

Patient	Pathological diagnosis	Site of primary lesion	Glucuronidase units Involved	Uninvolved	Site of lymph nodes	Glucuronidase units Involved		Uninvolved	
C.	Carcinoma	Breast	890	168	Ipsilateral axilla	1,960	3,250	816·	572
					Contralateral axilla	3,700		633	510
								365	645
K.	Carcinoma	Breast	3,650	143	Axilla	4,580	2,260	1,980	640
S.	Undifferentiated adenocarcinoma	Stomach	817	437	Lesser and greater curvature	1,522	1,565		
						1,455	1,533		
						1,457	1,085		
B.	Carcinoma	Colon	827	449	Mesentery	1,895	1,284	206	
T.	Malignant melanoma	Finger	....	....	Axilla	2,532		680	
G.	Lymphosarcoma	....	....	....	Axilla	1,730	....		
E.	Lymphoblastoma	Parotid	1,940	1,475		....	....	....	
W.	Adenocarcinoma	Prostate	....	....	Neck	6,700	....		
M.	Adenocarcinoma	Stomach	....	....	Lesser curvature	1,621	....		
L.	Undifferentiated carcinoma	Stomach	....	....	Hilum of lung	1,956	2,520	....	·
						2,520			

The findings reported in Table II are self-explanatory. The elevated enzyme values of the primary tumors were paralleled by similarly raised glucuronidase in the lymph nodes involved with metastatic cancer (Figs. 1 to 19). There seems to be no relation between this phenomenon with the location in the body of the tumor studied. In 2 cases of lymphosarcoma, 1 in the parotid gland and the other in an axillary node, there were also seen higher values than in the uninvolved lymph node.

The data reported in Table III were obtained in all but one case from surgically removed specimens where pan-hysterectomy was done for pelvic car-

activity than the myometrium and vagina. In the post-partum patient the myometrium was analyzed since no endometrium could be found. However, placental tissue contained β-glucuronidase activity in the same range as that seen in myometrial tissue. The lactating breast showed an activity much higher than that of the relatively normal breast tissue recorded in Table I.

DISCUSSION

β-Glucuronidase has been shown to be related to the metabolism of the estrogenic hormones (7). The enzyme in the uterus responds specifically to

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DESCRIPTION OF FIGURES 1 TO 6

Photomicrographs of sections shown in Figs. 1 to 4 were made from tissues of patients reported on in Table II.

FIG. 1.—Patient C. Breast uninvolved by carcinoma. Shows a group of small ducts lined by normal cuboidal epithelium, and to one side of these there is some collagenous connective tissue. Mag. × 290. Glucuronidase activity 168 units.

FIG. 2.—Patient C. Carcinoma of breast. Shows irregular large tumor cells arranged in small nests and short cords which loosely infiltrate a stroma of collagenous connective tissue. Many of these tumor cells have invaded lymphatic spaces. Mag. × 225. Glucuronidase activity 890 units.

FIG. 3.—Patient C. Uninvolved lymph node from contralateral axilla. Shows a lymph node which is free of tumor invasion. There is some increase in the lymphoid elements however. On one side part of a normal germinal center can be seen. Mag. × 250. Glucuronidase activity 365 units.

FIG. 4.—Patient C. Involved node from ipsilateral axilla. Shows a complete destruction of lymph nodal architecture and a replacement by nests of large epithelial tumor cells in an abundant connective tissue stroma. Mag. × 230. Glucuronidase activity 1,960 units.

FIG. 5.—Patient C. Contralateral axillary node partly involved. Shows a small nest of malignant epithelial cells in one corner of a lymph node. The remainder of the node shows only some lymphoid hyperplasia. Mag. × 235. Glucuronidase activity 633 units.

FIG. 6.—Patient C. Contralateral axillary node completely involved. Shows complete replacement of lymph node by a dense sheet of epithelial tumor cells. Mag. × 225. Glucuronidase activity 3,700 units.

FIGS. 1-6

FIGS. 7-11

Patient	Age	Tissue	Glucuronidase Units	Remarks
N.	36	Vagina	669	.
		Endometrium	3,640	
		Myometrium	1,370	
		Endocervix	3,600	———
S.	25	Placenta	1,030	Partum
R.	37	Breast	1,050	Postpartum
		Uterus	844	
M.	48	Vagina	295	Postmenopause
H.	51	Uterus	199	
		Vagina	50	.
V.	59	Ovary	291	Postmenopause
		Uterus	365	
		Vagina	147	

estrogenic substances (8) even when these are administered in amounts within the physiological range. It has been suggested that the enzyme functions to synthesize the glucuronic acid conjugate of the hormone as a step in its utilization by the tissue. This process is regarded as one of "metabolic conjugation" rather than one of detoxication (8).

The position of physiological equilibrium in the reactions supposedly catalyzed by β-glucuronidase *in vivo*, seems to be far over in the direction of synthesis, when one considers the excretion of steroid glucuronidase (2) and characteristic elevations in blood glucuronidase (3) which occur during human pregnancy. However, it is conceivable that under certain conditions glucuronide hydrolysis may take place to an extent greater than normal. In such an event, the biologically less active conjugate might produce a relatively much more potent cell growth-stimulating substance, the free steroid hormone. This concept possesses many obvious attractive

features in any consideration of mechanisms of estrogen transport and of estrogen action in the body, especially in view of the report that human blood estrogen apparently consists of a complex of estriol glucuronide with protein (10).

Observations made on the human female reproductive organs seem to be in line with the participation of β-glucuronidase in estrogen metabolism. Thus, the elevated glucuronidase activity in the lactating breast and in the uninvolved tissue of the breast with chronic cystic mastitis may be explained on the basis of prolonged estrin action. Moreover, the decline in human uterine and vaginal glucuronidase activity seen after the menopause resembles the picture seen in mouse uterus following ovariectomy (7).

The present observations strongly suggest that elevated β-glucuronidase may be characteristic of cancer cells, although a high glucuronidase value does not necessarily imply a malignant state. It should also be pointed out that the differences in enzymic activity noted (Table I) appear to be consistently more striking in carcinoma of the breast than those of any other organ. Fibroids, which can be produced experimentally by estrogen (9), do not possess elevated glucuronidase activities. It has been suggested (3, 7), as one possibility, that the elevated glucuronidase in malignant neoplasms may represent a metabolic response to estrogens or to some closely related substance.

Elevated glucuronidase activity in malignant neoplasms may be due to an actual increase in enzyme protein synthesis or to the presence of an activating substance or to the removal of the inhibitor(s). These same possibilities have been pointed out in the case where uterine β-glucuronidase has been elevated in response to estrogen injection (8).

In conclusion, there is little doubt that there is

DESCRIPTION OF FIGURES 7 TO 11

FIG. 7.—Patient K. Carcinoma of breast. Section of breast tumor. Shows an almost solid sheet of malignant epithelial cells with large hyperchromatic nuclei and many mitotic figures. These cells are growing in closely arranged wide cords separated by lymphatic spaces. There is practically no fibrous tissue stroma seen. Mag. × 235. Glucuronidase activity 3,650 units.

FIG. 8.—Patient K. Uninvolved axillary node. Shows only a slight degree of lymphoid hyperplasia without any invasion by tumor. Mag. × 315 Glucuronidase activity 1,620 units.

FIG. 9.—Patient K. Partially involved axillary node. Mag. × 12. Shows a lymph node which contains three different portions. (a) The largest oval portion has been

completely replaced by dense growth of tumor cells. (b) The triangular portion to the right consists of lymphoid tissue with only a few nests of cancer cells. (c) The remainder consists only of fatty tissue. Glucuronidase activity (a) 4,580. (b) 2,260. (c) 411.

FIG. 10.—Patient T. Axillary node involved by malignant melanoma. Shows a complete replacement of this axillary node by large polyhedral cells with dark nuclei. These cells are arranged in loose sheets in close approximation to blood vessels. Mag. × 245. Glucuronidase activity 2,532 units.

FIG. 11.—Patient T. Uninvolved node. Shows a section of lymph node with a mild lymphatic hyperplasia. There is no evidence of invasion by malignant melanoma cells. Mag. × 270. Glucuronidase activity 680 units.

a relationship on the one hand of β-glucuronidase activity to estrogen action, and on the other hand of β-glucuronidase activity to processes of malignant growth. However, it should be fully realized that further work will be necessary to decide whether these relationships are more than coincidental.

SUMMARY

Tissues from surgical specimens were assayed for β-glucuronidase activity. Striking elevations in activity were found in malignant neoplasms of the breast, stomach, colon, uterus, ovary, penis, and lung as compared with the adjacent uninvolved tissue. Metastases to lymph nodes and other organs likewise showed notable differences.

Vagina, uterus and ovary removed from women of various ages were assayed for β-glucuronidase activity. There was a marked decline in activity after menopause. Breast, uterus and placenta at parturition contained substantial enzymic activity. On the whole these findings are in agreement with previously demonstrated correlations between estrogen activity and β-glucuronidase activity.

The elevations in β-glucuronidase activity in the neoplasms were interpreted as a metabolic response of the tissue to estrogen or some other closely related substance.

ACKNOWLEDGEMENTS

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DESCRIPTION OF FIGURES 12 to 15

FIG. 12.—Patient S. Uninvolved stomach. Shows section of uninvolved stomach wall. There is some atrophic gastritis and increased lymphoid infiltration of the mucosa and muscularis. No evidence of cancer in this section. Mag. × 82. Glucuronidase activity 447 units.

FIG. 13.—Patient S. Carcinoma of stomach. Shows section of stomach wall infiltrated by cancer cells singly and in small nests. There is no differentiation and there is lack of cohesion of the malignant cells. They are growing wildly in a fibrous tissue stroma. Mag. × 250. Glucuronidase activity 817 units.

FIG. 14.—Patient S. Involved node. Shows section of lymph node from the lesser curvature which has been largely invaded by cancer cells. These cells resemble the primary stomach tumor in their undifferentiated type of growth and lack of cohesion. Mag. × 260, Glucuronidase activity 1,565 units.

FIG. 15.—Patient S. Involved node. Shows section of another lymph node from the greater curvature almost completely replaced by tumor. In this case the tumor cells are arranged in larger nests. The centers of these nests have undergone necrosis to give the tumor a pseudoglandular appearance. Mag. × 260. Glucuronidase activity 1,457 units.
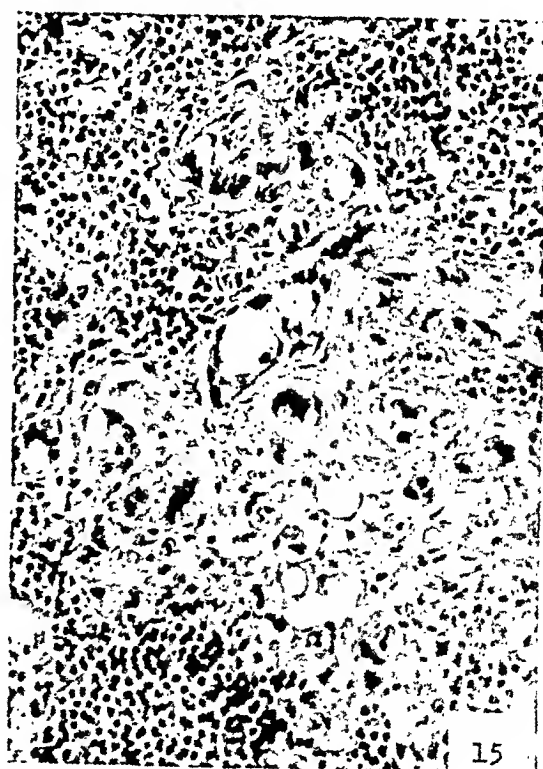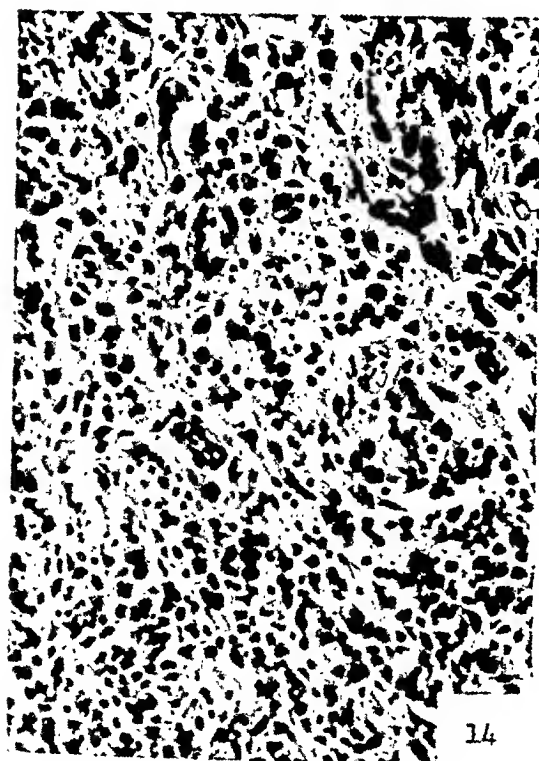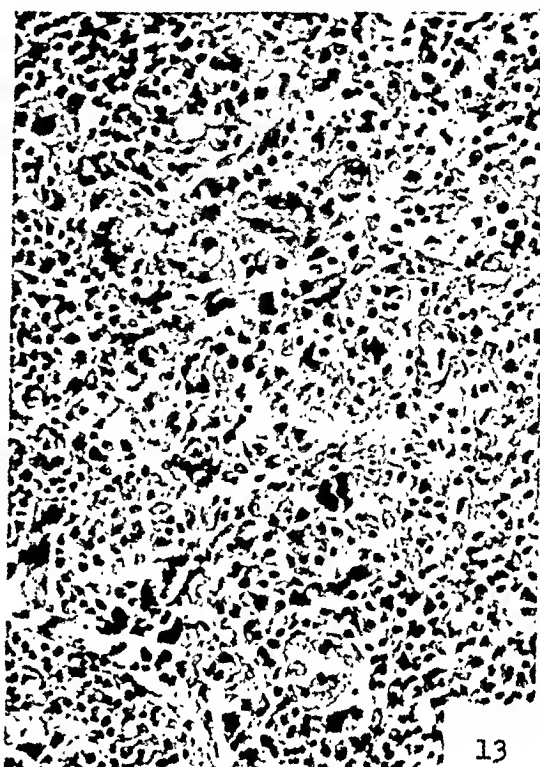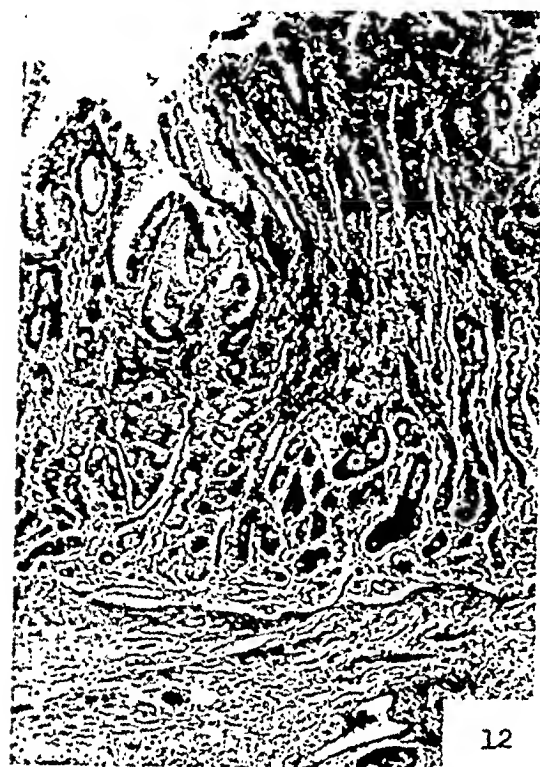
Figs. 12-15
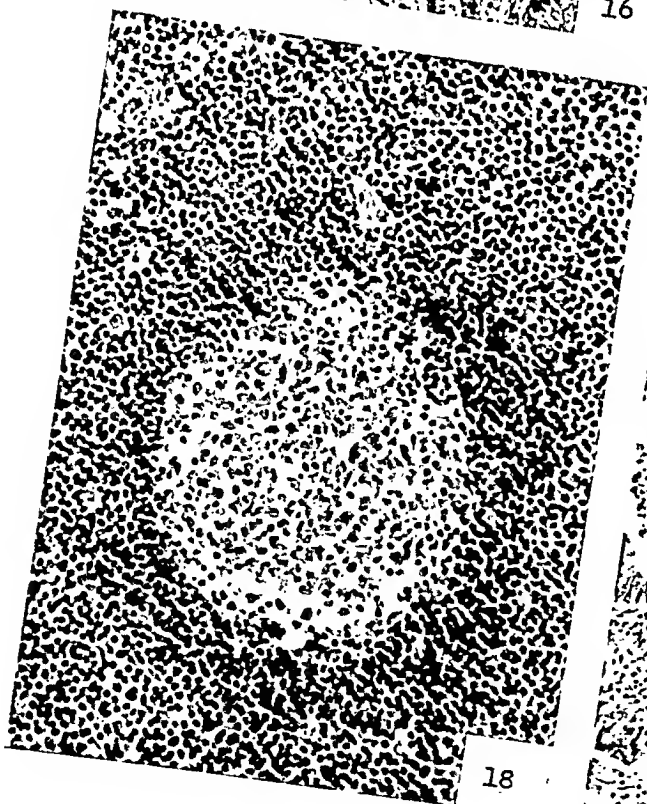
Figs. 16-19

DESCRIPTION OF FIGURES 16 TO 19

Fig. 16.—Patient B. Uninvolved colon. Shows a section of uninvolved colon wall with normal mucosa. There is some lymphocytic infiltration of the mucosa, but no evidence of tumor. Mag. ✕ 135. Glucuronidase activity 449 units.

Fig. 17.—Patient B. Carcinoma of colon. This shows section of colon wall with large columnar cells with hyperchromatic nuclei, forming irregular acini which invade the muscularis. The stroma surrounding these malignant acini is infiltrated with some lymphocytes. Mag. ✕ 225. Glucuronidase activity 827 units.

Fig. 18.—Patient B. Uninvolved perirectal node. This section shows only a slight lymphocytic hyperplasia. There is a normal germinal center seen. There is no evidence of malignant invasion in this node. Mag. ✕ 240. Glucuronidase activity 206 units.

Fig. 19.—Patient B. Involved perirectal node. This shows section of a lymph node completely invaded by tumor. The malignant cells are arranged in large irregular acini, in which the lumens contain much cellular debris. The surrounding fibrous tissue stroma is infiltrated by lymphocytes. Mag. ✕ 135. Glucuronidase activity 1,895 units.

Energy Mechanisms in Malignant Tumors in Relation to Chemotherapy*

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Differences in metabolism of neoplastic tissue and its normal homologue have been the subject of extensive experimental work. Burk (4) has suggested seven criteria of malignant metabolisms, while the studies of Greenstein (8) have done much to map the enzymatic patterns of malignant tumors. These studies as well as many others have pointed to the accentuation of both aerobic and anerobic glycolysis as well as to a diminution in the constituents and reactivity of the respiratory chain of enzymes viz., flavoprotein, cytochrome and cytochrome oxidase. Crystallization of these observations is found in the statement by Greenstein (8, p. 201) that "tumors tend to converge enzymatically to a common type of tissue."

The differences in metabolism between normal and malignant tissues have been the basis for many attempts to develop a chemotherapeutic approach to their control. Direct observation in tissue culture of differential sensitivity to various enzyme inhibitors was reported by Chambers, Cameron and Kopac (5).

Attempts to inhibit the glycolytic activity of tumor tissue *in vitro* have in some instances been encouraging (13, 3, 15). When given to the intact animal, however, these enzyme inhibitors in general have been found ineffective (12, 17, 11) except in a few instances (6, 7, 19).

The possibility of a new approach toward a rational chemotherapy of malignant tumors seemed to be indicated in terms of the energy-rich phosphate bond. The importance of these bonds in relation to cellular energy requirements has been stressed by Lipmann (14) and Kalckar (10). If malignant cells are particularly dependent on the glycolytic mechanism for their energy requirements it might be possible to inhibit their activity if one could selectively limit the formation or utilization of these bonds.

Based on this concept, an attempt was made to create a tentative model of biochemical function in malignant tissue as compared to the normal. It was felt that with the advent of malignant neoplasia there was a significant alteration in the energy mechanism of tissue. The preferred pathways would now involve the glycolytic mechanism while the respiratory enzyme reactions would be diminished or be of limited importance as compared to their role in normal tissue. It should be stressed that the respiratory functional potentiality is not absent but merely residual. This is of particular importance from the point of view of mechanisms of adaptation. The glycolytic mechanism itself may be divided into two main groups, the primary portion from triose to lactic acid and the tricarboxylic acid cycle which would serve as an accessory or secondary mechanism for energy production.

The value of any model or theory rests to a large extent on its ability to indicate significant experiments or to prognosticate and unify observations. On the basis of this hypothesis the following would be predicted.

1. *The preferred sites for inhibiting the primary glycolytic mechanism in order to obtain maximum destruction of energy-yielding reactions in malignant tissue.* These would occur at the points of creation of the high energy phosphate bonds, namely the coupled oxidation-phosphorylation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid, and the enolization of 2 phospho pyruvic acid to phospho-enol pyruvic acid. These reactions are inhibited respectively by iodoacetic acid and sodium fluoride.

2. *That such inhibition would have minimal effects on normal tissue.*

3. *That adaptation of malignant tissue to these inhibitors would occur. This would be associated with utilization of a secondary mechanism for energy production.*

(a) The tricarboxylic acid cycle is a likely secondary pathway. Inhibition of the cycle could be accomplished by the use of malonic acid which inhibits the succinic dehydrogenase system.

(b) Further adaptation after initial sensitivity of the malignant tissue to the glycolytic inhibitors would occur. This might well be accomplished

over the diminished metabolic pathways, that is, those using the respiratory enzyme chains particularly cytochrome oxidase. It should be recalled that these pathways are not absent but merely residual and could come to prominence when more favored reactions are blocked. Such reactions might be inhibited by the use of azide which blocks oxidase activity.

The schematic condensation of the broad groups of metabolic reactions as well as the sites of action of the inhibitors mentioned are indicated in Fig. 1.

4. *The various inhibitors, particularly the glycolytic, would be equivalent in terms of high energy phosphate bonds.* Given the acute lethal dose of the inhibitors and the therapeutic dose of one of them it should be possible to compute the therapeutic doses of the others.

The acute lethal doses were obtained from the data of Handler (9) who used intraperitoneal injections in rabbits. He obtained the following values for the various inhibitors: sodium fluoride 250 mgm. kgm., sodium iodoacetate 80 mgm. kgm., sodium malonate 1,500 mgm. kgm., sodium azide 10 mgm. kgm. For our calculations the value of 320 mgm. day was chosen for the average therapeutic dose of sodium fluoride for an adult. This was determined by studies of the therapeutic effects on leukemia cases to be described below. From these figures simple ratios were set up in the following form.

$$\frac{\text{Lethal dose X}}{\text{Lethal dose Y}} = \frac{\text{Therapeutic dose X}}{\text{Therapeutic dose Y}}$$

The values for iodoacetic acid and malonic acid were determined in this manner. In the case of sodium azide the value arrived at was divided by 3, in view of observations that the cytochrome oxidase content of tumors is roughly in a 1:3 ratio with normal tissue (8, p. 249). The comparisons of the calculated doses with the doses found to be clinically effective are listed in Table I.

TABLE I: COMPARISON OF CALCULATED AND CLINICALLY EFFECTIVE DOSES OF VARIOUS INHIBITORS

Inhibitor	Calculated dose*, mgm.	Clinically effective dose*, mgm.
Sodium fluoride	——	320
Iodoacetic acid	92	60-90
Malonic acid	1892	1000-1500
Sodium azide	2.7	1.8-2.5

* Dose/day for average adult.

This report gives the results of preliminary experiments based on the concepts mentioned above. The various inhibitors were tested on cases of acute leukemia as well as various types of malignant tumors. Acute leukemia was chosen initially as a particularly good subject for study in view of the uniformly poor prognosis and the ease of following therapeutic effects by means of peripheral blood counts. While there are reports of occasional spontaneous remissions in acute leukemia, these are rare. Further, the remissions are not predictable as to time and method of occurrence. The essential validity of the foregoing predictions is indicated by the beneficial therapeutic effects observed in a significant number of the patients treated.

The various drugs were given orally in divided doses. In the case of sodium fluoride it was necessary to prevent the formation of hydrofluoric acid in the stomach. This was achieved either by the use of enteric coated tablets[1] or by the simultaneous administration of amphoteric antacids.

Acute myeloblastic leukemia.—Ten cases were studied, 5 of which showed definite clinical and hematological improvement, 2 showed some improvement coincident with therapy, while 3 experienced little or no effect. Improvement, when it occurred, consisted of gain in strength, appetite and alertness, and decrease in blasts, adenopathy and hepatosplenomegaly.

Of particular interest was the case of L. G., a 3½ year old white girl. Administration of sodium fluoride and iodoacetic acid (together) was accompanied by disappearance of blasts from the peripheral blood. Adenopathy and hepatosplenomegaly disappeared within a month. Following cessation of therapy for 2 weeks, there was a recurrence of the original symptoms and adenopathy. Resumption of treatment with sodium fluoride and iodoacetic acid failed to bring any regression in the adenopathy or hepatosplenomegaly, or to improve the clinical status. It was assumed that the cells had become adapted to these inhibitors and were now using an accessory pathway of metabolism. The tricarboxylic acid cycle was considered to be the next mechanism most likely to be available. The use of malonic acid was decided upon because of its known ability to inhibit the succinic acid dehydrogenase system. Within a week of the institution of malonic acid, in conjunction with the other two inhibitors, the glands became soft and fluctuant, and were resorbed. After about a month, during which all three inhibitors were administered, there was a recurrence of hepatosplenomegaly, glands and blasts. The child died shortly thereafter from pulmonary hemorrhage, apparently due to a lack of platelets. The total length of observation was 5 months.

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[1] Kindly furnished by Endo Products, Inc., Richmond Hill, New York.

It is particularly noteworthy that although the glycolytic inhibitors were effective initially, a process of adaptation appeared to take place so that the initial sensitivity was lost. It was felt that adaptation might have been accomplished by the use of reactions involving the "diminished metabolic pathway" as indicated in Fig. 1. Alteration in the leukemic cell was also suggested by the observation that morphological changes occurred in

as indicated by recurrence of adenopathy and elevation of the white count, it was noted that a transition had occurred from the original blast cell to a cell which looked very much like an unusual lymphocyte. It varied in size from 8 to 20 μ in diameter and was characterized by an almost complete lack of cytoplasm. The nucleus possessed a condensed chromatin pattern in which nucleoli were either absent or reduced to small cresentic

Fig I
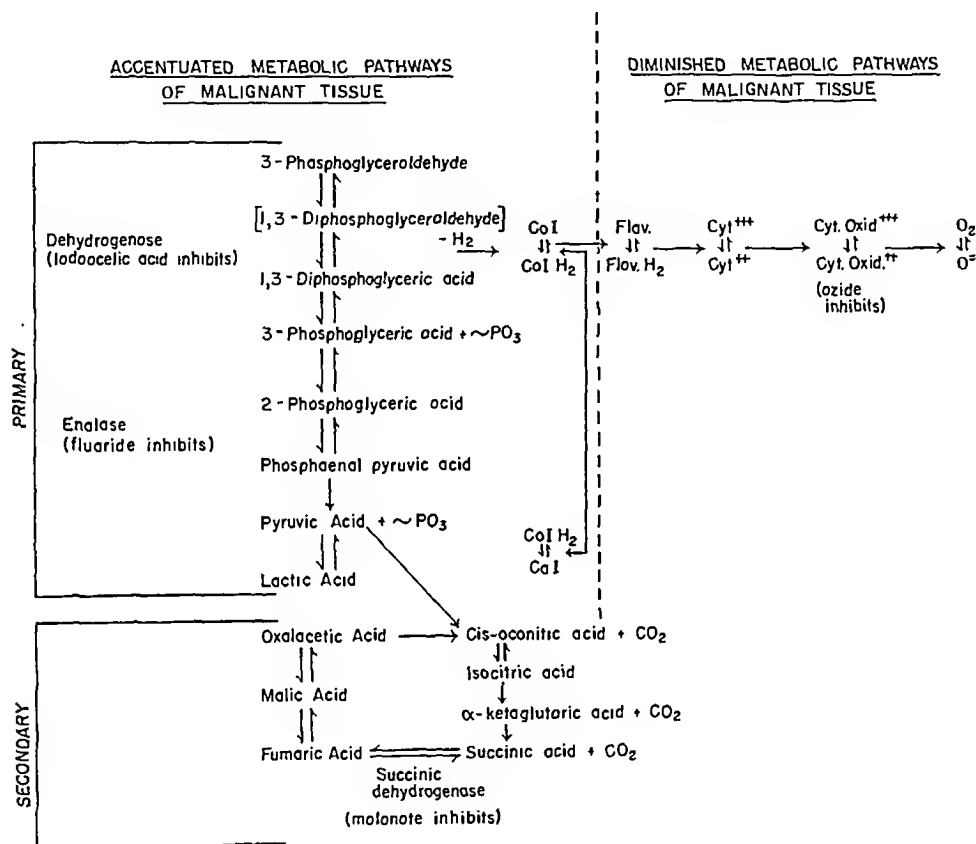
FIG. 1.—Schematic condensation of major metabolic pathways in malignant tissue.

the leukemic cell after exposure to the glycolytic inhibitors coincident with the development of resistance to these drugs. The original myeloblastic cell usually possesses a well defined basophilic cytoplasm, and a vesicular nucleus which contains several distinct nucleoli. Leukemia patients having such cells were almost uniformly sensitive to the glycolytic inhibitors, sodium fluoride, iodoacetic acid and malonic acid. Treatment resulted in disappearance of adenopathy, hepatosplenomegaly and elimination of these malignant cells from the peripheral blood. However, when adaptation occurred,

structures. These variations between the original cell type and the adaptation cell are illustrated in Fig. 2 and 3.

This relationship between metabolic sensitivity and morphological appearance of the leukemic cell is well illustrated by the following protocol.

S. R., (Fig. 4) 35 year old white male developed inguinal adenopathy in October, 1946. Biopsy was interpreted as lymphosarcoma; the peripheral blood count was normal. The patient received x-ray therapy to the groin and in addition was started on the glycolytic inhibitors. In spite of

continuous exposure to these drugs the patient seemed to be declining slowly. On December 24, 1946 a typical picture of acute myeloblastic leukemia was noted in the peripheral blood. This was corroborated by sternal-marrow aspiration. The malignant cells at this time were almost equally divided between two types, (a) a myeloblastic cell having well defined cytoplasm and a vesicular nucleus containing nucleoli, and (b) the adapta-

extensive and continued therapy with the glycolytic inhibitors. At the same time the clinical course was slowly down-hill, with progressive adenopathy. It seemed likely that in the face of the glycolytic inhibitors the malignant cells were using the diminished metabolic pathways which, as indicated in Fig. 1, involve oxidase activity. Therefore an attempt was made to evaluate the effect of an inhibitor of oxidase activity, namely, sodium azide.

FIG. 2.—Blood smear from case of acute myeloblastic leukemia. 2 large cells showing prominent nucleoli and well defined cytoplasm are typical myeloblasts. These cells are sensitive to the glycolytic inhibitors.

FIG. 3.—Blood smear from case of acute myeloblastic leukemia, after treatment with the glycolytic inhibitors. These cells are adaptation cells and are not sensitive to glycolytic inhibitors. Azide can induce reversal of adaptation cell to typical myeloblast.

tion type cell described above. In spite of continuous exposure to the glycolytic inhibitors (sodium fluoride 80 to 160 mgm. t.i.d., iodoacetic acid 30 mgm. t.i.d. and malonic acid 0.5 gm. t.i.d.) the patient showed slow but progressive development of adenopathy and hepatomegaly, the continued presence of malignant cells in the peripheral blood smear, absence of polys and general debilitation. At the same time it was noted that the malignant cells were becoming predominantly of the adaptation type. As can be seen from Fig. 3, there was little or no effect on the malignant cells, despite

The administration of the glycolytic inhibitors was discontinued on February 13 and sodium azide in doses of 0.625 mgm. t.i.d. started on February 17. This was continued for 10 days, during which time there seemed to be a slight decrease in adenopathy as well as some fall in the malignant cell count. The azide was then stopped and the glycolytic inhibitors reinstituted in the following doses: sodium fluoride 80 mgm. q.i.d, iodoacetic acid 30 mgm. b.i.d. and malonic acid 0.5 gm. b.i.d. In the interim between the cessation of the azide therapy and the reintroduction of an effective concentration

of the glycolytic inhibitors there was a marked increase in the malignant cell count and adenopathy. There was a coincident reappearance of nucleoli and basophilic cytoplasm in the blast cell. This was followed by an increase in polys and disappearance of malignant cells and adenopathy. Omission of therapy was again followed by reappearance of clinical discomfort and adenopathy and leukemia cells. These cells were of the original type, show-

tors were reintroduced, following which decrease in the blasts and adenopathy was again noted. The patient died as the result of hemorrhagic tendencies due to lack of platelets. The total time of observation was 6 months.

It should be mentioned that to almost all of our leukemia patients supportive transfusions were administered. Although there are reports that in some cases this alone may result in temporary re-

S.R. - Acute Myeloblastic Leukemia
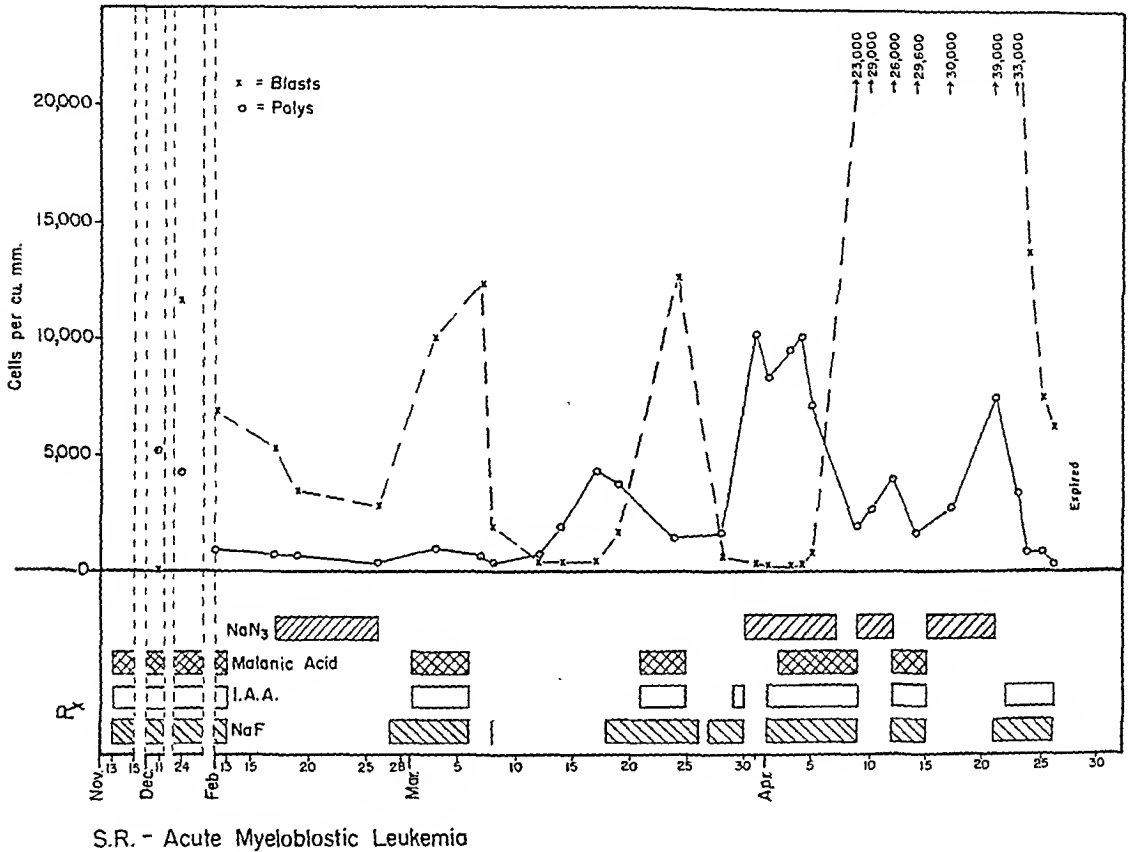
Fig. 4

FIG. 4.—The effect of the various inhibitors on case of acute myeloblastic leukemia.

ing nucleoli and cytoplasm. Re-introduction of the glycolytic inhibitors was again followed by clinical and hematological remission lasting several weeks. However, the malignant cells reappeared in spite of exposure to the azide and glycolytic inhibitors. Now they were predominantly of the adaptation type and little beneficial effect of the combined drugs was noted. However, after preliminary exposure to sodium azide alone, the percentage of the adaptation type of cell fell, while the original cell type reappeared. At this point the glycolytic inhibi-

mission, we found no correlation between any transfusion (more than 200 given) and clinical or hematological remission.

A rather uniform clinical pattern was observed in response to the therapeutic agents. Thus, clinical effect was not usually manifest until after three weeks of therapy. In the leukemia cases, however, some change in the blood count was usually noted as early as 24 hours after start of sodium fluoride therapy. This consisted of a decrease in the blasts either through drop in white count or appearance

of increased percentage of smudges with an associated drop in percentage of blasts. The exact mechanism was not predictable although it appeared that the blasts with abundant cytoplasm usually became smudges before the count dropped. This response may be so marked that the smudges may constitute as much as 85 per cent of the total count (Fig. 5).

Associated with the rapid destruction of the leukemic cells there is an elevation of temperature, which may go as high as 106° F. This usually lasts from 5 to 7 days; when it falls the adenopathy and hepatosplenomegaly are usually gone and the blood cleared of blasts. If the count previously consisted almost entirely of leukemic cells, there will be a marked leukopenia. Whether or not there will be return of the normal constituents depends on the bone marrow function. In general, the bone marrow response appears to be inversely proportional to the duration of the disease. So long as the blood remains free of malignant cells there is little evidence of the usual clinical features of agranulocytosis. In fact, the patient is usually clinically quite well.

The occurrence of hyperpyrexia associated with a therapeutic effect as seen in leukemias is usually not seen in other types of malignant neoplastic disease receiving this type of therapy.

Acute lymphatic leukemia.—Of 4 cases treated, all showed definite clinical and hematological improvement. One case treated with sodium fluoride alone underwent progressive changes, coincident with therapy, to what appeared to be a low grade chronic leukemia. She remained clinically well and active for 2 years. There was then a recurrence of the original acute type. Unfortunately circumstances prevented testing efficacy of the medication at this time. The patient succumbed.

Acute monoblastic leukemia.—In the single case studied, little change could be noted in the progressive decline over a period of 5 weeks' treatment with sodium fluoride, iodoacetic acid and malonic acid.

Gastric carcinoma.—Two cases were treated, both of which showed definite clinical improvement. In one, the large immobile epigastric mass initially present was converted within 5 weeks to a freely movable mass definitely smaller in size. The 70 year old patient died a cardiac death within 2 months. The other patient could not tolerate the medication after 4 weeks and therefore no effective doses were administered after that period. Death occurred from progression of the disease within 4 months.

Lymphosarcoma.—Coincident with treatment for 5 weeks, a retroperitoneal lymphosarcoma about the size of a man's head shrank to the size of a baseball. This dramatic lessening in mass was accompanied by clinical improvement. There followed a 2 week period of rest from therapy, during which the tumor again increased in size; subsequent resumption of therapy for 10 days was associated with a second decrease. An exploratory
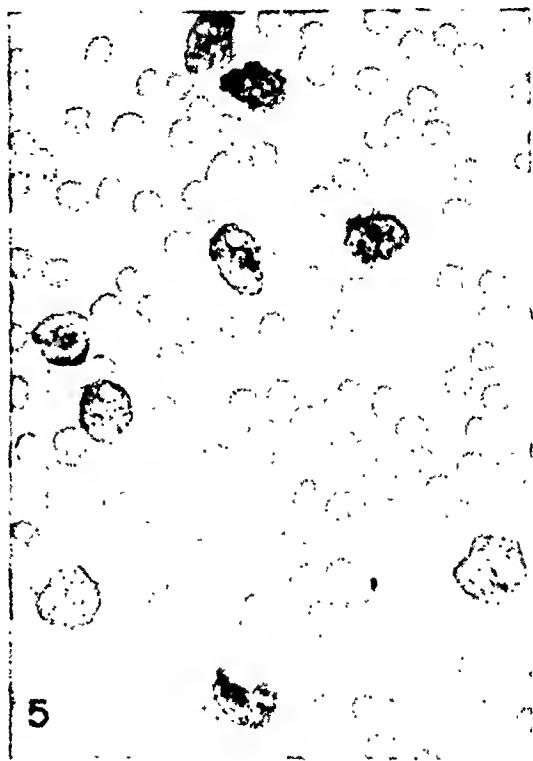
Fig. 5.—Blood smear from case of acute myeloblastic leukemia, showing extensive destruction of the blasts by the glycolytic inhibitors.

operation was now performed. The patient died of a retrograde peritonitis subsequent to the operative procedure. Surgical biopsy and postmortem microscopic examination revealed extensive and unusual necrosis and replacement fibrosis.

Carcinoma of the colon with metastasis to the liver.—Three cases showed a similar tendency for initial improvement in clinical status and some shrinkage of the enlarged livers within a month. However, these patients exhibited an inability to tolerate the medication beyond that period, experiencing nausea and vomiting. This was soon followed by a downward trend.

Carcinoma of the lung.—Four cases were studied

and all showed improvement coincident with therapy. Observations included decrease of pleural effusion, gain in weight and other signs of clinical improvement. One of these cases is still under observation, the others have died.

The following condensed protocol will serve to illustrate the findings in the former case of lung carcinoma.

W. K., 65 year old white male, having a long standing history of chronic bronchitis. Starting in November, 1946, the patient noted increasing weakness and distress. X-ray revealed atelectasis at the base of the right lung. Bronchoscopy disclosed a mass in the bronchus which proved to be squamous cell carcinoma. There had been a progressive weight loss of 17 pounds from November to December.

The patient was first seen by us on December 30, 1946, at which time he weighed 110½ pounds. He was emaciated, had a chronic cough, poor appetite and gasping respiration. Hemoglobin was 9.0 gm. He was given all three glycolytic inhibitors, sodium fluoride, 80 mgm. t.i.d.; iodoacetic acid, 30 mgm. b.i.d.; and malonic acid, 0.5 gm. b.i.d. In addition he was started on a series of injections of testosterone propionate, 25 mgm. t.i.d.

Under this form of treatment there was a progressive gain in weight, strength and hemoglobin, so that by March 26, 1947 he weighed 124 pounds and had 14 gm. of hemoglobin.

Some loss of weight and reappearance of cough recurred which was checked by administration of sodium azide followed by the glycolytic inhibitors.

The patient died in June, 1947. Autopsy revealed that extensive purulent material in the atelectatic right lung had spilled over and occluded the left bronchus.

The use of testosterone in conjunction with the inhibitors in this case does not seem to have altered the general pattern of response to the inhibitors. The effects were neither accelerated or deterred. Further, the dose in this case was less than the dose of testosterone usually employed.

Carcinoma of the testis.—Two cases were treated. The principal complaint of one patient was excruciating pain of sciatic type arising from metastasis in the right psoas area, proved by x-ray. Bed rest and opiates were of little avail. Within a month this patient was well enough to be ambulatory; he refused further treatment because it made him nauseous. (Both he and his wife were unaware of the diagnosis.) After a temporary period of well-being, his clinical status deteriorated rapidly. The second patient showed decrease in gynecomastia, relief from pain and increased strength. The 24

hour output of 17-ketosteroids decreased from 29.2 mgm. to 20.5 mgm. This patient had also an advanced case of diabetes, and died suddenly in what appeared to be diabetic coma. The total duration of study was 3 months.

Hodgkins' disease.—Three cases were treated, all of whom showed definite clinical improvement. In 2 of the cases, where there was marked adenopathy, the glands were seen to shrink strikingly under therapy consisting of the glycolytic inhibitors. Parallel clinical improvement was noted in appetite, strength and loss of cough and itching. The results in one of the cases is illustrated in the following protocol.

J. F., 32 year old white female, admitted to the Brooklyn Cancer Institute on February 2, 1947 with a 2 year history of adenopathy starting after delivery of a normal child. The adenopathy had increased strikingly for the past 7 months, particularly in the left cervical region. Examination revealed extensive cervical adenopathy which surrounded the neck in a collarlike fashion, completely effacing the chin line. There were also extensive axillary adenopathy and hepatosplenomegaly. Chronic non-productive cough was present. X-ray revealed marked swelling of the left neck shoulder angle and considerable enlargement of both hilar, more prominent on the right. The upper mediastinum bulged to the right. W.B.C., 40,000 (polys 76 per cent), hemoglobin, 12.9 gm. Sternal puncture revealed hyperplasia of the poly series, with no evidence of primary hematological disorder. The methylene blue reducing time, according to the method reported by one of us (M. M. B.), was 18.5 minutes (1).

After 3 weeks of treatment with the glycolytic inhibitors the glands receded markedly, the methylene blue reducing time dropped to normal and the W.B.C. decreased. Insensitivity to the medication occurred and was reversed by administration of sodium azide for a period of 1 to 2 weeks. This phenomenon of reversal of insensitivity has been accomplished 3 times thus far.

At present, after about 6 months of therapy, the patient feels better, the glands are smaller and the cough is gone.

It is stressed that the agents used, and in the dosage employed, had no apparent effect upon normal cells. Thus in all cases of carcinoma treated with the same doses as the cases of leukemia there was no morphological change in any of the blood components. Similarly there was no apparent effect of inhibitor sodium fluoride on the clinical course or hematological picture in 4 cases of chronic myelogenous leukemia.

DISCUSSION

The therapeutic response observed, as well as the striking similarity between the calculated dose and the actual dose needed to produce therapeutic effects, lends support to the validity of the original thesis upon which these studies were undertaken.

It is also significant that the observations reported here give insight into the mechanism of adaptation by malignant cells. Although the phenomenon of adaptation still constitutes a major limitation in this and other chemotherapeutic modalities, it appears that it does not involve the creation of enzymatic mechanisms *de novo* but rather the utilization of previously existent secondary pathways. This would be in agreement with the studies of Sevag (20) with bacterial adaptation to chemotherapeutic agents. The adaptation found by us appears to follow a preferential and step-wise pattern. Thus the preferred pathway appears to involve the glycolytic cycle from the triose to lactic acid. On this basis the tricarboxylic acid cycle would serve as an accessory or secondary mechanism for energy production. Reactions involving azide-sensitive enzymes (cytochrome oxidase, peroxidase, catalase) may be considered as of limited importance as compared to their role in normal tissue. However, their functional potentiality is not absent but merely residual, and comes to prominence when more favored reactions are blocked. It should also be mentioned that some leukemic cells, which originally were similar in morphology to the adaptation cell described above, failed to show any sensitivity to the glycolytic inhibitors.

Correlation between biochemical activity and morphological alterations has long been a problem of acute interest to the cellular physiologist. Thus the apparent change in cell type in response to the glycolytic inhibitors poses new problems on the cytoplasmic localization of enzymes and the function of nucleoli. Much work is needed along these lines to clarify the full significance of these observations.

The studies reported by Stowell (20) on the relationship between nucleolar size and cytoplasmic nucleoprotein production in the liver cell is of great interest in this connection. He reported direct relation between nucleolar size and increase of cytoplasmic nucleoprotein.

The use of glycolytic inhibitors in the intact individual requires that particular attention be paid to the possibility of deleterious effects on the normal tissue of the retina and the intestinal mucosa, since both tissues have been reported as having metabolic activity similar to neoplastic tissue, particularly in regard to a high glycolytic activity. In our studies there have been no detectable impairment in vision, nor any change in gastrointestinal function or gross or microscopic alternation which would indicate appreciable damage. Either the metabolism of the intact retina is not similar to neoplastic tissue or it adapts very readily and with more facility than does malignant tissue.

The recent reports of Rosenthal (16) indicate that aerobic glycolysis of an intensity characteristic of cancer tissue is not a normal metabolic feature of the mucosa of the small intestine.

The need for further work with the therapeutic agents described is unquestioned. Of particular interest would be the incorporation of these simple molecules into the structure of a larger organic group so as to impart increased and more lasting effect. Thus arsenic, although toxic to the spirochete, requires a particular organic carrier to achieve maximal therapeutic and minimal toxic effect.

The data considered in this paper involve consideration of specific sensitivity of malignant tissue. However, the factor of the body substrate in the control or spread of tumors should not be overlooked. It is common clinical experience to find that similar tumors pursue widely different courses in different individuals. In this connection the variation induced in the reducing power of plasma of patients with malignant neoplastic disease is important. The beneficial therapeutic effect of sulfhydral compounds reported by one of us (M. M. B.) (2) appears to be a valuable adjunct in the chemotherapeutic armamentarium. Further use of the sulfhydryl compounds in cancer patients has indicated the possibility of actual tumor inhibition and regression in addition to the relief of symptomatic complaints. These results would appear to be secondary to alteration or stimulation of body defense mechanisms rather than the result of direct effect on the tumor. The sulfhydryl compounds in present use include glutathione and cysteine. It appears advisable to extend observations to include compounds of di- and tri-thiol structure.

Study of the effects of the combined action of inhibitors and sulfhydryl compounds now in progress give indication of enhanced therapeutic effects.

SUMMARY

Differences in metabolism between normal and malignant tissues have been the basis for many attempts to develop chemotherapeutic measures. A new approach toward the chemotherapy of malignant tumors seems indicated in terms of the energy-rich phosphate bond.

Based on this concept, an hypothesis of biochemical function in malignant tissue as compared with normal was formulated as follows: With the advent of malignancy there is a significant alteration in the energy mechanism of tissue. The preferred pathways involve the glycolytic mechanisms while the respiratory enzyme reactions are diminished as compared with their role in normal tissue. The respiratory functional potentiality is not absent but merely residual. The glycolytic mechanism itself may be divided into (a) the primary portion from triose to lactic acid, and (b) the tricarboxylic acid cycle which serves as a secondary mechanism for energy production.

Based on this hypothesis the following were used in the treatment of various types of human malignant neoplastic diseases: sodium fluoride, iodoacetic acid, malonic acid and sodium azide. The essential validity of the foregoing hypothesis is indicated by the beneficial therapeutic effects observed in a significant number of patients treated.

ACKNOWLEDGEMENT

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International Cancer Research Commission*

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This Commission will promote cooperation in cancer research throughout the world. It will therefore supply the mechanism for teamwork which, though widely needed, has hitherto been halting and without organization. As now established, the Commission is the first international group devoted exclusively to research on cancer. This activity is broadly conceived to include clinical, laboratory and statistical investigations, indeed all efforts to expand knowledge of this disease to the end that improvements will be made on a large scale in prevention, diagnosis and treatment of cancer. Systematically to bring in cancer workers of all nations is clearly indicated. With different cultural backgrounds and different habits of mind, they may well view the same problem from different angles and try to solve it in different ways.

FORMATION

Conditions were especially favorable for the birth of the Commission at the International Cancer Research Congress held in St. Louis, September 2 to 7, 1947. The Congress was sponsored by the Union Internationale Contre le Cancer and by the American Association for Cancer Research. The attendance from abroad was further increased by the action of the United States Department of State in transmitting invitations to foreign governments to send official delegates. The time was ripe for such a meeting, because we are entering a period of almost worldwide reconstruction. Cancer facilities destroyed in the war must be rebuilt and enlarged. Investigations interrupted must be resumed and new researches started with greater hope of success. Obviously it is advantageous to take up this fight for light on the mysteries of cancer armed with full knowledge of what others have accomplished in the war years and plan now to do. It is also important to utilize the most effective apparatus which has become available. Then, too, secrecy is being lifted from scientific discoveries made in researches bearing on the war. Information about these may help to shape cancer research problems. The eagerness of those attending the Congress to spare no pains in the overcoming of obstacles was matched by their determination to pool their resources and go forward together.

President Truman, in his opening message to the Congress, said: "This meeting has large significance to the United States and to the world at large from every point of view—social, economic, political and spiritual. No further argument is needed to support such a statement than the fact that in the United States alone 180,000 persons die annually of cancer. The last war showed more clearly than ever before the value of coordinated research. How fruitful, therefore, will be this assembly from the whole world of scholars interested in the solution of the cancer problem."

In a later telegram the President advised the Congress "that it is now possible for the United States to take an important forward step toward greater international cooperation in the field of medical and biological research. On behalf of the people of the United States I am pleased to announce to the Fourth International Cancer Research Congress that progress in the production of radioisotopes by the United States Atomic Energy Commission now permits limited distribution to qualified research workers in other countries of radioisotopes principally for medical and biological research. I know that the representatives of the United States attending the cancer research congress share my hope that the open, impartial and truly international character of medical research will carry over into the realm of other problems of world concern. The sharing by and among all nations of both the means and the results of cancer research will reduce the loss of life and human suffering from disease throughout the world."

With such encouragement and with deepest personal feelings of the urgency of the cancer problem, the Congress set to work framing suggestions for international cooperation. Within a very short space of time history was made in the creation of the International Cancer Research Commission consisting of a single representative of the following nations attending the Congress:

Argentina: JUAN ESTEBAN PESSANO, Tucumon 1694, Buenos Aires.

Australia: R. KAYE-SCOTT, 105 Collins St. Melbourne.

Belgium: J. MAISIN, University of Louvain, Louvain.

Bolivia: HECTOR FERNANDEX FERRUFINO, Hnos. Machego No. 908, La Paz.

Brazil: ANTONIO PRUDENTE, Benjamin Constant 171, Sao Paulo.

Canada: G. E. RICHARDS, Ontario Cancer Treatment & Research Foundation, Toronto.

Chile: A. RAHAUSEN, Dept. of Experimental Medicine, Avenida Irarrazaval 849, Santiago.

China: TU-SHAN JUNG, Dept. of Radiology, Peking Union Medical College, Peking.

Columbia: RUBEN A. GARCIA, Instituto del Radium, Bogota.

Czechoslovakia: H. SIKL, Dept. of Pathology, University Charles IV, Prague.

Denmark: J. ENGELBRETH-HOLM, Universitetets Patologisk-Anatomiske Institut, Frederik V.s Vej 11, Copenhagen.

Duchy of Luxembourg: SIMON HERTZ, c/o Nathan Hertz, Grand 'rue et rue du Fosse, Luxembourg.

Egypt: JOSEF N. AZZOUNI, St. Mary's Hospital Group, 1325 S. Grand Ave., St. Louis, Mo. (acting for Official Delegate).

El Salvador: DON RICARDO POSADA, Universidad de el Salvador, San Salvador.

France: A. LACASSAGNE, Laboratoire de Biologie, Institut de Radium de l'Universitede Paris, Due d'Ulm 26, Paris.

Great Britain: ALEXANDER HADDOW, Royal Cancer Hospital, Fulham Road, London, SW3.

Greece: EFSTATHIOS G. MINOPOULOS, Director General, Athens Anti-Cancer Institute, Athens.

India: VASSONT R. KHANOLKAR, Director of Laboratories, Tata Memorial Hospital, Bombay.

Iran: CHARLES OBERLING, Dean, Medical School of Teheran, Teheran.

Iraq: SALMAN FAIK, Assoc. Prof., Royal College of Medicine, Bagdad.

Italy: FRANCESCO PENTIMALLI, Instituto Pathologia General, San Andrea Dame 8, Naples.

Korea: IL SUN YUN, Dept. of Pathology, Seoul University Medical School, Seoul.

Mexico: IGNACIO MILLAN, Director of Tumor Clinic, General Hospital, Avenida Vera Cruz 69, Mexico, D.F.

Netherlands: R. KORTEWEG, Laboratorium Antoni Van Leeuwenhoek-Ruis, Sarphatistraat 108, Amsterdam.

Nicaragua: FERNANDO VALEZ PAIZ, Manuaga, D.N.

Norway: LIEV KREYBERG, Dept. of Pathology, University of Oslo, Oslo.

Palestine: A. HOCHMAN, Hebrew University, Jerusalem. (Memorial Hospital, Physics Dept., 444 E. 68th St., New York City.)

Republic of Panama: ERNESTE ZUBIOTA, P.O. Box 1595, Panama City.

Peru: EDUARDO CACERES, Dept. of Anatomy, San Marcos University, Lima. (Chicago Tumor Institute, 21 W. Elm St., Chicago, Ill.)

Philippine Republic: JUAN A. ARCELLANA, College of Medicine & Philippine General Hospital, University of Philippines, Manila.

Portugal: MANUEL PINTO, Institute de Oncologia, Lisbon. (1324 Eutaw Place, Baltimore, Md.)

Siam: CHANAI RUANGSIRI, Chulalankarana University, Bangkok.

Sweden: ERIK ASK-UPMARK, University of Upsala, Upsala.

Switzerland: H. R. SCHINZ, Rontgenistitut & Radiotherapeutische Linic, Kantonsspital, Zurich.

Regency of Tunisia: CHARLES W. ANDERSON, Directeur Du Laboratoire de Cancerologia de l'Institut des Hautes Etudes de la Regency, Tunis.

Turkey: PERIHAN CAMBEL, General Secretary, Turkish Association for Cancer Research, Ankara.

Union of South Africa: LT. COL. WEINBREN, SAMC, National Cancer Association of South Africa, Johannesburg.

United States of America: E. V. COWDRY, Prof. of Anatomy, Washington University, St. Louis, Mo.

Uruguay: FELIX LEBORGNE, Ibicuy 1210, Montevideo.

Venezuela: HERMOGNES RIVERO, El Ministro de Sanidad, Caraces.

Representatives from Austria, Cuba, Equador, Paraguay, Java, Hungary and the Union of Soviet Socialist Republics, though named and expected, did not attend.

Ethiopia, Lebanon, Dominican Republic, Eire, Finland, Saudi Arabia and Yemen, Afghanistan, Liberia, Costa Rica, Honduras, Poland, New Zealand and Bulgaria indicated interest in international cooperation in cancer research.

The 138 representatives from countries other than the United States included official delegates sent by their countries in response to the invitation transmitted to them by the U. S. Department of State, as well as many acknowledged leaders in cancer research who had no official status.

An organization meeting was held on September 2 by these representatives from abroad, together with the following official delegates to the Congress appointed by the President of the United States:

JOHN J. BITTNER, Minneapolis, Minnesota.

JAMES P. COONEY, Colonel U. S. Army, Washington, D. C.

E. V. COWDRY, St. Louis, Missouri.

W. U. GARDNER, New Haven, Connecticut.

RONALD L. GRAND, Commander U. S. Navy, Washington, D. C.

ELISE L'ESPERANCE, New York, N. Y.

C. P. RHOADS, New York, N. Y.

LEONARD L. SCHEELE, National Cancer Institute, Washington, D. C.

SHIELDS WARREN, Boston, Massachusetts.

RICHARD B. WILLIAMS, Commander U. S. Navy, Washington, D. C.

Since the group was evidently too large to operate effectively and some nations had numerically much larger representation than others, it was unanimously decided, after full discussion, to assign the duty of making recommendations to a smaller group consisting of but one representative from each of the 40 nations. This group was designated as the Executive Committee of National Representatives.

The Executive Committee held meetings on September 3, 4 and 5. A representative from each of three countries, the United States, Great Britain, and Mexico, successively presided over these meetings. The recommendations prepared were presented for approval on September 6 in English, French and Spanish to the larger body of National Representatives, which established the executive committee, and they were approved. Later in the same day the recommendations were submitted to the entire Fourth International Cancer Research Congress and were adopted unanimously and enthusiastically. These recommendations follow:

A representative body, consisting of one member elected by each of the 40 national groups represented at the Congress, advises the creation of an International Cancer Research Commission.

This group of national representatives recommends that the Congress agree upon the following principles:

1. That cancer research include all efforts to advance our knowledge of cancer by clinical, experimental or other means.

2. That the Commission consist of one member from each country here represented, with equal voting power. Other nations not here represented will be welcome on the same basis.

3. That the principal source of financial support should be governmental.

4. That the governments should be invited each to send one national national representative who should be actively engaged in cancer research, to

serve for a period of three years, and advises that such representation be changed at the end of this three years. Further, that the Governments after two years select an additional representative who will serve without vote prior to succeeding the voting nominee.

5. That the Commission should arrange meetings once a year, and never consecutively in the same country.

6. That an executive committee be constituted, made up of five members and not more than seven as follows, with alternates:

> *1 from Asia,*
> *1 from Latin America,*
> *1 from United States of America,*
> *2 from Europe.*

7. That the first executive committee be constituted as follows:

LATIN AMERICAN:

Member	Alternate
Dr. Millan (Chairman)	*Dr. Lebornge*

UNITED STATES:

Dr. Cowdry	*Dr. Gardner*

ASIA:

Dr. Khanolkar	*Dr. Tu-Shan Yung*

EUROPE:

Dr. Maisin	*Dr. Lacassagne,*
Dr. Haddow	*Dr. Engelbreth-Holm*

8. That the Commission form part of the Union Internationale, replacing the Camite Scientifique. It advises that this Commission, within the organization of the Union Internationale, be given the greatest possible freedom of action to attain its goal, and that it have direct power to solicit and distribute financial aid, which has been agreed to by the Union.

9. That this Fourth International Research Congress requests of Governments that the group of national representatives here assembled and its Executive Committee be permitted to serve as the first appointee.

ORGANIZATION

Since the purpose of the Commission is to cooperate effectively in cancer research on a worldwide front, the principle of decentralization was accepted as basic.

Thus, the Executive Committee is broadly representative and the agreement that no member of the Commission shall serve for more than 3 years will prevent gradual domination by a few people and will regularly spread the responsibility to others. The headquarters of the Commission, now in

Mexico City under the direction of Dr. Ignacio Millan (Avenida Vera Cruz 69), will move to the country of his successor in 1950. If, for some reason, his alternate, Dr. Leborgne, has to take his place before 1950, headquarters will be shifted to Montevideo, Uruguay, until Dr. Leborgne's retirement.

Meetings of the Commission will be held annually, but never twice consecutively in the same country since that would tend to promote what we are trying to avoid—centralization. In choosing the location of meetings the strength of nations in cancer research will not be the determining factor. It was agreed that the meeting can sometimes do more good when held in a country actively striving to organize cancer research than in one already well advanced in this respect.

As has been indicated, there is only one Commission member for each nation. These members have equal voting power irrespective of the size of the nations they represent. The value of the Commission depends solely upon free and regular exchange of opinion and mutual helpfulness among its members and between the sovereign nations they represent. There is and can be no element whatever of control by the Commission as a body.

This restriction of members to a minimum will reduce the cost of meetings to a coverage of the bare essentials. It will also call for greater care in the selection of members. The opinion has been repeatedly expressed that the members should be actively engaged in cancer research, that they should be nominated by national civilian organizations and that they should be appointed by governments. Indeed the demand for this modus operandi may lead to the formation of National Cancer Societies in countries not now so equipped and to the strengthening of such societies already operating.

DUTIES

Many of the duties of the Commission are self-evident: (1) to strengthen the position of the charter members of the Commission representing the 40 member nations, (2) to arrange for the 20 other nations who formally expressed interest in the International Cancer Research Congress to appoint members to the Commission and (3) to include on the same basis Japan, Germany and Spain.

A report on the Commission has been sent by the U. S. Department of State to all of the nations originally invited to appoint official delegates to the Congress. This will add dignity to the members and the cause in which they are working. In addition, the Chairman of the Commission, ably sup-

ported by those who attended the Congress who are in fact ambassadors of good will, will labor in the same direction. Thanks to these ambassadors, and in no small measure to the American Cancer Society, which assigned to the Congress an expert in Publicity, Mr. Patrick McGrady, the educated public in many lands is beginning to look to the Commission for progress in the fight against the common enemy, cancer.

So much depends on the support given by the public of each country to its national representative on the Commission that publicity looms large in the service rendered by the Commission. The Commission as a world group will give such assistance to its members when invited to do so; but never will it interfere by giving unsolicited advice. In some instances, descriptive pamphlets already available require only translation into other languages before they can be distributed as desired by the members. In other cases they must be modified or completely rewritten to suit the local conditions. To guide such publicity directed to the achievement of world cooperation in cancer research would be an inspiring task for the right person. It must be well managed because a few mistakes would undermine the influence for good of the Commission.

Already the Commission is in receipt of many welcome suggestions as to how it can best operate. Some are general in character while others are specific. The Chairman, Dr. Ignacio Millan, will take them all under advisement. It is his duty to reach a decision on his own responsibility, or, in case of doubt, to refer them to his Executive Committee or to the whole Commission at some annual meeting. The same applies to other important questions besides service.

The Commission is ideally constituted for integration by the pooling of information at the annual meetings to which the members will bring their hopes and plans for cancer research in their several countries and from which they will return encouraged and strengthened by consultation with others dedicated to the same cause. It would be helpful if each member consented to make a brief progress report and if all of these reports could be combined and published as an annual world progress report and if all of these reports could be combined and published as an annual world progress report on cancer research. The fellowship developed at the meetings should grow between meetings by mutual exchange of news, especially about new technics and apparatus, new research projects, and possibly concerning better means of diagnosis and treatment.

To be most helpful the principle of two-way exchange should be fostered. For all paths to lead in one direction promotes centralization, rather than decentralization, with spreading of cooperation. Travel for cancer research between nations should be more nearly equal going and coming. For example, it is helpful for investigators to visit England from Argentina; it is also advantageous for them to visit Argentina from England. Travel should be to all countries, as well as from all countries, in drawing the world together for more effective cancer research.

Research would be facilitated if there could be some agreement on the nomenclature of malignant and benign tumors for at present there is a good deal of confusion. The American Medical Association, the American College of Surgeons and the American Society of Clinical Pathologists have a committee now working on this subject. A member of this committee has approached the Commission with the idea that we want not a national nomenclature but a world nomenclature. Before adoption of the revised system of names, it should therefore be referred to the whole International Cancer Research Commission at one of its annual meetings. Preliminary discussion would facilitate matters.

It is possible that the Commission might be able to devise a standard form for recording the clinical history of cancer patients acceptable to all of its member nations. Widespread use of this form would be of enormous assistance in cancer research because observations on cancer in different geographic areas offering a wide variety of climatic, racial, nutritional and other important factors would then have been made on the same basis. In a word, the results would stack up constructively.

Allied to these problems of nomenclature and clinical histories is that of Tumor Registries. A strong committee on Tumor Registries has been appointed by Dr. Shields Warren of the Atomic Energy Commission. He has expressed himself as very sympathetic to the proposition that, if possible, the plans of organization should be so adjusted that the Commission will be able to recommend them for world adoption. This is, of course, a difficult task involving much friendly discussion with Dr. Millan and his associates, but it is very definitely worthwhile.

It has been proposed that the Commission proceed further in laying the basis for cancer research, not only by discussion and by reaching international agreements, but also by the actual contribution of microscopic specimens. According to this plan, preparations would be made of recognized types of human malignant and benign tumors and of so-called precancerous lesions. These would be of high technical excellence. Other preparations would show microchemical technics as applied to cancers. Collections of such standard specimens would be supplied to the member nations wanting them. Like all activities of the Commission, this service if undertaken, should be cooperative. Instead of having a central laboratory do the job, several members might be willing to share the work so that the headquarters of the Commission would only have to assemble the collections and distribute them.

Not infrequently cancer research is held up by lack of some chemical, dye, or piece of apparatus. The worker feels frustrated and may even abandon very promising investigations. When thus inhibited the various members of the Commission strategically situated throughout the world should act as agents for the member in difficulty, or his friends, by advising him where the missing material or equipment can be secured with a minimum of delay. Such requests for aid should be channeled through the Chairman of the Commission.

Much time can also be lost in cancer research by the investigation of side lines of little real significance for want of expert advice. Workers pride themselves in their independence and they tend often to learn the hard way by repeated failures. Having in mind the grim character of the killer, cancer, speed is needed. Therefore, help should be sought and the question is, where can it best be secured? The haphazard and often inadequate way is to write to one's friends. In this matter I think the Commission can develop great usefulness. It might for instance decide to construct a master plan of cancer research which would include the names of a few leaders actively engaged on all fronts. Having done so, the Chairman of the Commission would then be in a position immediately to answer inquiries by sending the name and address of the person best qualified to advise wherever he may happen to be. This information is well known to mature investigators of long experience working in great centers of cancer research, but it is not known to beginners and to those more or less isolated geographically from others dedicated to the same objective. It is the high purpose of the International Cancer Research Commission to bring laborers in this field of cancer together and to bolster teamwork.

FINANCIAL SUPPORT

There are two immediate financial needs of equal urgency: To provide for the first annual meeting

of the Commission and to supply headquarter's expenses.

At present there are 40 charter member Nations. By next summer, when the meeting will be held, it is expected that about 10 other Nations will have designated members. Travel expenses must then be obtained somehow for representatives of 50 Nations. Certainly, some of these countries will pay the necessary expenses, whether from governmental or private funds. Others will obviously be unable to do so. The old selfish attitude would be to say, "Well, leave them out." But to act in this narrow way would be greatly to cripple the usefulness of the Commission. Consequently, it becomes the privilege of the more fortunate nations to help these others financially, with the understanding that as soon as possible they will defray the expenses of their own representatives at future annual meetings. How many these will be is not known definitely—perhaps 30. Reckoning at $1,500 each, which was the amount usually allowed for travel to the Fourth International Cancer Research Congress in St. Louis, the sum to be raised for the first annual meeting of the Commission amounts to $45,000.

The Chairman and members of the Commission serve for a maximum of 3 years without salary from the Commission. As they are busy people with other duties to perform, the Headquarters of the Commission needs to be strengthened by a full-time salaried staff not subject to the 3 years limitation, because it is important for some of them to carry on in the administration of subsequent chairmen in other parts of the world for the sake of continuity. This staff should consist of a Vice-Chairman, a Director of Publicity, 2 secretaries, and translators paid per diem. The annual cost of salaries would be in the neighborhood of $30,000.

Expenses of headquarters are difficult to estimate. Offices and furnishings might well be loaned by the institution to which the Chairman belongs. Other items include materials, printing, postage, etc., plus a sum for travel between meetings of the Commission. The last named item is important because some of the member nations should be visited and a meeting of the 5 member executive committee would be essential. Such expenses would amount annually to about $20,000.

Evidently, therefore, a total of about $100,000 is required properly to launch this the first International Cancer Research Commission. This would permit operation for somewhat more than a year because the personnel could not be immediately assembled and at the beginning expenditures would be small but before the second annual meeting of the Commission, we hope further enlarged, additional money would be needed. By that time a more extensive sharing of the expenses by member nations would be achieved.

The problem is how to raise the initial $100,000. The mechanism has been established. The receipts thus far small and altogether unsolicited ($1,490.72) have been deposited by the Treasurer of the Commission, Mr. E. S. Jones, in the First National Bank in St. Louis. Disbursements will be made on vouchers signed by the Chairman and another member of the Commission and periodic audits will be made for the Commission. In this country the National Advisory Cancer Council of the United States Public Health Service is legally unable to make grants for travel and it is doubtful whether the American Cancer Society can properly aid financially any activity outside of the United States. We believe that merely making known in the member nations the purpose, organization and activities of the International Cancer Research Commission will bring in adequate financial support, since thinking people everywhere understand the need for cancer research on a world-wide basis.

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